GENE EXPRESSION PROFILING IN ISCHEMIC AND NONISCHEMIC CARDIOMYOPATHY

by

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Abstract

Background Despite our growing understanding of the pathophysiology and management of heart failure, there exist no strategies to individualize therapy using predictors of long-term prognosis and response to therapy. Gene expression analysis using microarray technology provides a phenotypic resolution not possible with standard clinical criteria and could offer insights into disease mechanisms and also identify markers useful for diagnostic, prognostic, and therapeutic purposes. Thus, the two major applications of this technology are gene discovery and molecular signature analysis. These two applications were explored in studies involving the two major forms of cardiomyopathy, ischemic and nonischemic (ICM and NICM, respectively).

Methods For a gene discovery analysis, we compared the gene expression of 21 NICM and 10 ICM samples with that of 6 nonfailing (NF) hearts using Affymetrix U133A microarrays and Significance Analysis of Microarrays software. For molecular signature analysis, we identified and validated an etiology signature with Prediction Analysis of Microarrays software using 48 ICM and NICM myocardial samples obtained from different institutions and at different clinical stages.

Results The gene discovery analysis demonstrated that compared to NF hearts, 257 genes were differentially expressed in NICM and 72 genes in ICM. Only 41 genes were shared between the two comparisons and an analysis of the gene subsets revealed shared and unique disease-specific gene expression between end-stage cardiomyopathy of different etiologies. The molecular signature analysis demonstrated that an etiology prediction profile accurately distinguished between ICM and NICM, was generalizable to

samples from separate institutions, specific to disease stage, and unaffected by differences in clinical characteristics.

Conclusions We have demonstrated that there are shared and distinct genes involved in the development of heart failure of different etiologies, and that a molecular signature can accurately identify etiology in cardiomyopathy. These findings highlight the utility of the two distinct applications of gene expression analysis, and support ongoing efforts to develop cause-specific therapies and expression profiling-based biomarkers in heart failure. The ultimate goal is individualized therapy, whereby a heart failure patient could, through gene expression analysis, be offered an accurate assessment of prognosis, and how individualized medical therapy could affect his or her outcome.

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Kittleson MM, Hare JM. Molecular signature analysis: The potential of gene expression analysis in cardiomyopathy. *Future Cardiology* 2005; 1: 793-808. With permission from Future Medicine, Ltd.

Kittleson MM, Minhas KM, Irizarry RA, Ye SQ, Edness G, Breton E, Conte JV, Tomaselli G, Garcia JGN, Hare JM. Gene expression analysis of ischemic and nonischemic cardiomyopathy: Shared and distinct genes in the development of heart failure. *Physiol Genomics* 2005; 21: 299-307. With permission from the American Physiological Society.

Kittleson MM, Ye SQ, Irizarry RA, Minhas KM, Edness G, Conte JV, Parmigiani G, Miller LW, Chen Y, Hall JL, Garcia JGN, Hare JM. Identification of a Gene Expression Profile that Differentiates between Ischemic and Nonischemic Cardiomyopathy. *Circulation* 2004; 110: 3444-51. With permission from Lippincott Williams and Wilkins.

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*Adapted from:

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The current state of heart failure management

Heart failure therapy

The incidence of heart failure is rapidly increasing in the United States and is a major cause of morbidity and mortality.[1] Over the past two decades, there have been remarkable advances in medical and surgical therapies designed to improve the symptoms and survival of patients with heart failure, including angiotensin-converting enzyme (ACE) inhibitors,[2-4] beta-blockers,[5-8] aldosterone antagonists,[9;10] angiotensin-receptor blockers,[11-13] cardiac resynchronization therapy,[14-16] implantable defibrillators,[17-19] and ventricular assist devices.[20]

However, it is still not clear which patients will benefit most from which therapies, and a better understanding of the differences in response to therapy is essential because there are an increasing number of interventions that may be costly, such as implantable cardiac defibrillators;[21] risky, such as ventricular assist devices;[20] or scarce, such as donor hearts for cardiac transplantation.[22]

In addition, the issue of polypharmarcy is looming. Although the number of medications shown to improve mortality in heart failure is growing, the more medications an individual takes, the greater the risk of adverse effects.[23] While large-scale clinical trials have shown overall improvements in quality of life and survival for heart failure patients, there are clearly subsets of patients who respond differently to therapies. For example, ACE inhibitor intolerance is more common in patients with ischemic cardiomyopathy (ICM),[24] milrinone has a deleterious effect in ICM but not nonischemic cardiomyopathy (NICM),[25] amiodarone may prevent sudden death only in NICM, [26;27] and the benefit of combination therapy with isosorbide dinitrate and hydralazine may be

greater in African-American heart failure patients.[28] Thus, the ability to individualize therapy is critical, so that patients receive only those therapies from which they will derive benefit, while avoiding those which might lead to harm.

Heart failure prognosis

Within the spectrum of heart failure, prognosis varies widely [29]. Although clinical indicators of poor prognosis exist, including increasing age;[30] decreased ejection fraction;[31] higher New York Heart Association functional class;[32] diabetes mellitus;[33] worsening renal function;[34] decreased sodium;[35] increased B-type natriuretic peptide;[36] and increased uric acid;[37] none have been shown to assist in tailoring the rapidly increasing array of pharmacologic, device, and surgical therapies for heart failure patients.

Gaps in our understanding of heart failure

Which newly diagnosed cardiomyopathy patients will improve their ejection fraction and functional status, and which will go on to develop circulatory collapse and require cardiac transplantation, is still beyond our ability to assess. Furthermore, we currently cannot determine which patients will benefit most from therapies with improvement in symptoms and survival, and which patients will have little benefit or even harm. Gene expression analysis using microarray technology, by providing a phenotypic resolution not possible with standard clinical criteria, could provide better information regarding prognosis and response to therapy in heart failure patients.

Microarray analysis

Overview of microarray technology

With the emergence of microarray technology, it is now possible to simultaneously assess the expression of tens of thousands of gene transcripts, with

remarkable resolution of phenotypic characterization. Microarrays rely on the principle of complementary hybridization of nucleotide sequences and have taken advantage of robotic technology to create platforms as small as a few inches wide containing thousands of DNA sequences (Figure 1.1A).

There are two types of microarrays. The first type, traditionally known as a cDNA microarray, utilizes relatively long multimers of probe cDNA (500 – 5000 bases), is made primarily in individual institutions, and is often organ- or disease-specific. Examples include the CardioChip from Brigham and Women's Hospital[38] and the LymphoChip from Stanford University.[39] Usually, two samples are studied on a single array, and relative gene expression is measured.

The second type of microarray, known as an oligonucleotide microarray, uses smaller DNA probes (20 – 80 bases) and has a number of advantages: 1) it is commercially produced by companies such as Affymetrix; Agilent, Amersham, or AME Bioscience, and thus the quality control is standardized and the cost of production is minimized; 2) it contains an unbiased genome-wide rather than organ system-specific set of probes; 3) it contains more gene transcripts (almost 50,000 on the newest Affymetrix 2.0 GeneChip Array); 4) it measures absolute, as opposed to relative, gene expression because individual samples are studied on separate arrays.

Both cDNA and oligonucleotide microarrays consist of a solid support, usually a glass slide or a nylon membrane, onto which DNA sequences are attached. Using high-speed robotics, thousands of DNA sequences are either spotted using pins or an ink-jet printer or synthesized directly on the array using PCR or photolithography. For a microarray experiment, RNA from a sample is extracted, transcribed into cDNA, labeled

with fluorescence (more common) or radioactivity, and then hybridized to the microarray. Complementary sequences remain bound to the array. Expressed genes are identified by the position of the corresponding bound probes on the microarray and their abundance is determined by the intensity of the measured signal (Figure 1.1B). Thus, an experiment with a single microarray can provide information on the expression thousands of genes simultaneously: the transcriptome of a given tissue sample can be determined.

The next essential step in microarray analysis is assurance of adequate array quality. All arrays should be subject to quality control. For example, if Affymetrix gene chips are used, there are parameters established by the manufacturer for assessing sample and array quality.[40] These parameters include measurement of 260/280nm ratios of both total RNA and biotinylated cRNA to assess for RNA purity; visual inspection for the presence of image artifacts; and assessment of average background and noise value, exogenously added prokaryotic hybridization controls, percent present calls, scaling factors, and internal control genes, usually actin and glyceraldehyde phosphate dehydrogenase (GAPDH). Only arrays in which all parameters are within acceptable levels should be subject to further analysis.

The final step in microarray preparation is conversion of probe set data into gene expression levels. Because each gene on a microarray is represented by multiple probes, the hybridization intensity of all these probes must be taken into account in determining the overall expression of each gene. While many methods exist, the principles are the same and involve three steps: background and signal adjustment, data normalization, and summarization of probe information into gene expression levels. Background and signal adjustment corrects for background noise, adjusts for cross hybridization, and ensures

that all values fall on the proper scale. Normalization eliminates systematic differences between arrays, either using a reference array or the combined information from all the arrays. Finally, summarization involves converting the multiple probe hybridization intensities on the array into a single measure of gene expression. These background-corrected, normalized, and converted probe summaries of gene expression can then be used for microarray analysis.

Applications of microarray analysis

The challenge in microarray experiments is in the experimental design and statistical analysis, where the number of variables assessed is orders of magnitude higher than the number of individuals studied. There is concern about the dangers of data-mining, where lists of thousands of genes are generated without an understanding of the technical and statistical pitfalls of microarray analysis. To avoid this, it is essential to understand the distinct applications of this technology.

Because the state of the transcriptome in a given diseased tissue may contain a highly accurate representation of key biological phenomena, patterns of gene expression have potential to provide insights into disease mechanisms and also to identify markers useful for diagnostic, prognostic, and therapeutic purposes. Thus, the two distinct major applications of this powerful technology are gene discovery and molecular signature analysis, and these two applications have different goals, statistical methods, and validation strategies (Table 1.1).

Gene discovery

Overview

Gene discovery focuses on identifying differentially expressed genes characteristic of different disease states, through which novel genetic pathways and potential therapeutic targets may be elucidated. In the field of cardiomyopathy, many microarray studies have focused on gene discovery. Studies have offered insights into the differential gene expression of failing and nonfailing hearts,[41-49] dilated and hypertrophic cardiomyopathy,[50] and before and after left ventricular assist device (LVAD) support.[51-55]

Statistical methods

Many statistical methods have been used to identify differentially expressed genes. All methods, however, rely on the same principles: comparing expression between two or more groups by taking into account the magnitude of the difference between groups and the variability of expression within groups while adjusting for multiple comparisons. The latter point is essential in microarray analyses, where the number of variables (i.e., thousands of genes) is orders of magnitude greater than the number of subjects.

Significance Analysis of Microarrays (SAM)[56] is one approach to identify differentially expressed genes. SAM uses a modified t-test statistic to determine the observed and expected relative differences in gene expression. To calculate the observed relative difference in gene expression, $d(i)$, the difference in average gene expression between the two groups is divided by the standard deviation of repeated measures of gene expression, $s(i)$ and a constant, s_o to minimize the impact of genes with low levels of expression. The expected relative difference is the average of the relative difference of

all possible permutations of the samples. The observed relative difference in gene expression is then plotted against the expected relative difference, and this scatterplot is used to determine which genes are differentially expressed. Differentially expressed genes are those displaced from the line of unity by a distance greater than the chosen threshold, and the threshold is chosen to minimize the false discovery rate.

Thus, SAM identifies genes with statistically significant changes in expression by identifying a set of gene-specific statistics (similar to the t-test, thus taking into account both magnitude of change and variability of expression) and a corresponding false discovery rate (similar to a p value adjusted for multiple comparisons). Another common statistical method for identifying differentially expressed genes is the ANOVA test, used by commercially available software such as GeneSpring from Agilent.

Validation

Once differentially expressed genes are identified, the transcript abundance is routinely confirmed by a complementary method, such as quantitative PCR, Northern blotting, or RNase protection assays.[57] In cardiomyopathy, studies have relied mainly on quantitative PCR, with over 80% agreement in all studies with the results of microarray hybridization.[41-50;52;53;55;58] Less commonly, levels of the corresponding protein have also been measured, with less agreement between transcript and protein abundance.[53;54] However, this is not surprising, since a number of factors affect the measured protein abundance, including differences in mRNA localization, processing, stability, translation efficiency, as well as posttranslational protein modification and interactions.

Failing versus nonfailing hearts

Despite differences in sample size and statistical analyses, there is congruence between studies aimed at identifying differentially expressed genes between failing and nonfailing hearts.[41-49] Significantly regulated genes across all studies are mainly those belonging to functional categories of cell growth and maintenance, cytoskeleton/sarcomere, metabolism, and signal transduction. This agreement across studies offers further validation for studies of differential gene expression.

However, these studies have a number of limitations, including their focus on solely binary comparisons (failing versus nonfailing) and concentrating mainly on NICM. These limitations have limited their applicability and highlighted the need for more sophisticated analyses incorporating ICM samples along with samples of NICM and nonfailing hearts.

Impact of left ventricular assist device support

A number of studies have also examined the changes in gene expression that occur following LVAD support.[51-55] Through mechanical unloading of the failing ventricle, LVAD support results in beneficial hemodynamic, neurohormonal, structural, and biochemical changes, termed reverse remodeling.[58] This phenotypic alteration, coupled with the availability of tissue samples obtained at the time of implantation, has offered a unique opportunity to study the transcriptomal shifts associated with reverse remodeling. Many of these analyses have demonstrated alterations in genes involved in vascular signaling, including downregulation of neuropilin-1, a vascular endothelial growth factor receptor,[55] upregulation of endothelial nitric oxide synthase,[52] and upregulation of the APJ receptor for apelin, an endogenous cardiac inotrope present in the

cardiac vasculature.[53] Thus, there is congruence in these studies as well, and different studies have all identified significant alterations in genes that regulate vascular organization and endothelial function in response to mechanical unloading of the failing human heart.

The most sophisticated of the analyses of LVAD-supported hearts compared the gene expression of nonfailing hearts with that of failing hearts with and without LVAD support to identify adaptations that represent normalization of gene expression.[54] Of the 3088 transcripts that were differentially expressed in failing relative to nonfailing hearts, only 238 actually demonstrated a consistent response to LVAD support and of these, more than 75% demonstrated persistence or exacerbation of their heart failure expression pattern after LVAD support. This suggests that the alterations in gene expression following LVAD support are distinct from a return toward normalcy and may not represent a simple reversal of changes observed during disease progression.[54] These findings offer unique insights into the nature of LVAD-associated reverse remodeling and could theoretically be clinically useful in identifying individual patient responses to mechanical unloading.

Implications for molecular signature identification

Although the majority of microarray analyses in cardiomyopathy to date have focused on gene discovery, these studies nevertheless provide insight into the feasibility of molecular signature analysis. In two studies, heart failure of different etiologies demonstrated different patterns of gene expression in unsupervised analyses. An unsupervised analysis of gene expression does not take into account *a priori* definitions, such as clinical parameters of etiology or disease stage, in the division of samples into

groups. Rather, a global assessment of gene expression alone is used to determine the relatedness of samples, and the significance of the grouping is then assessed. In one study, the NICM samples demonstrated more extensive global changes in gene expression than ICM samples following LVAD support.[51] In another study, the overall gene expression of familial and alcoholic cardiomyopathy was distinct from that of idiopathic cardiomyopathy.[48]

Heart failure patients of different clinical stages also exhibit different patterns of gene expression in unsupervised analyses. In one study of failing and nonfailing hearts, a distinct cluster of patients who were of the highest medical urgency status awaiting cardiac transplantation emerged in an unsupervised analysis.[46]

These findings suggest that gene expression can be correlated with clinically relevant parameters in heart failure patients. However, because these studies focused on gene discovery, the observations could not be applied prospectively to identify and validate a gene expression signature to distinguish subjects based on these parameters, thus emphasizing the need for studies focused solely on molecular signature analysis.

Molecular signature analysis

Statistical methods

The goal of molecular signature analysis is to identify a pattern of gene expression that is associated with a clinical parameter, such as etiology, prognosis, or response to therapy, thus potentially providing greater diagnostic or prognostic precision than that currently available from standard clinical information. There are a number of methods that can be used for molecular signature analysis, including partial least squares regression, neural networks, and shrunken centroids, and all rely on the same basic

principles.[59-61] First, samples are divided into groups based on a clinically relevant parameter, such as disease etiology, prognosis, or response to therapy. Then a molecular signature is created by choosing genes whose expression is solidly associated with the parameter in question, by weighting genes based on their individual predictive strengths.

Prediction Analysis of Microarrays (PAM) is one approach in molecular signature analysis.[62] PAM uses the method of nearest shrunken centroids to identify and validate the smallest set of genes whose expression is associated with a predefined class. A standardized class centroid is the average expression of each gene in a given class divided by the within-class standard deviation. The overall centroid is the average expression of each gene in all classes divided by the pooled within-class standard deviation. Standardization allows greater weight to be given to genes whose expression is stable within a class. PAM creates shrunken centroids by shrinking the class centroids towards the overall centroid by a threshold amount. Thus, the shrunken centroids are "de-noised" versions of centroids that act as prototypes for each class. The threshold is chosen by 10-fold cross validation. In this process, gene expression prediction profiles with different numbers of genes (i.e., from different thresholds) are fit on the basis of 90% of the samples and then tested on the remaining 10%. This process is executed multiple times, and the output is the error rate using different thresholds. The prediction profile is chosen as the smallest list of genes that can be used to predict the classification of an individual sample with the minimal error rate.[62;63]

To then classify independent samples, PAM computes each test sample's squared distance from each of the class centroids. The predicted class is the one whose centroid is closest to the expression profile of the test sample. In contrast to SAM and other

methods of identifying differentially expressed genes, PAM focuses mainly on the stability of gene expression and on the smallest number of genes required to create a molecular signature.

Validation

While the goal of gene discovery is to identify differentially expressed genes which offer insight into novel genetic pathways or cause-specific therapies, the goal of molecular signature analysis is to identify a pattern of genes that differentiates between clinical entities with a precision not possible based on standard clinical information. Thus, the identity of the mRNA transcripts in the signature or whether they are translated into protein may or may not have immediately discernable bearing on the utility of the pattern.[63] Therefore, the validation strategy is also unique: testing the accuracy of the identified molecular signature in samples distinct from those used to create it.

Application of molecular signature analysis to neoplastic disease

In neoplastic disease, molecular signature analysis can determine prognosis and response to therapy. In breast cancer, a molecular signature was identified that predicted disease outcome in young patients with breast cancer better than standard clinical and histological criteria: a poor prognosis signature was associated with a 5-fold increased risk of distant metastases in 5 years, a difference that would justify early intensive adjuvant chemotherapy.[64] In large B-cell lymphoma, a molecular signature predicted survival better than standard clinical methods.[65] Similar results have been obtained for acute myeloid leukemia[66;67], chronic lymphocytic leukemia,[68] prostate cancer[69;70] and CNS tumors.[71]

These advances in neoplastic disease have shown that molecular signature analysis can augment current standard practices to better individualize management and choose among treatment options. It is essential to determine if the powerful predictive power of molecular signature analysis demonstrated in neoplastic disease can be applied to cardiovascular disease.

Research plan

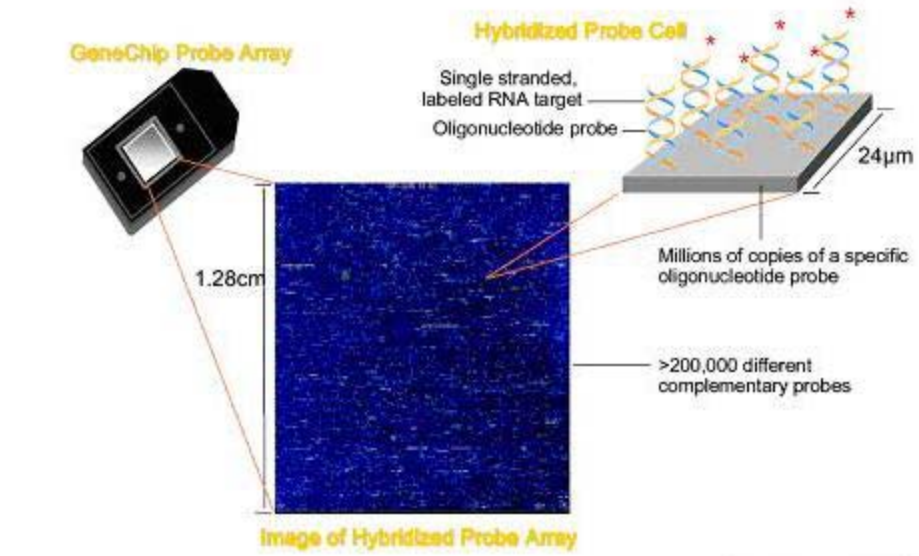
Clearly, gene expression analysis in cardiomyopathy is at its earliest stages. The current dissertation utilizes the two major applications in gene expression analysis, gene discovery and molecular signature analysis. First, while many studies have focused on gene discovery, most have involved small samples sizes and comparisons between two groups, such as failing and nonfailing hearts and before and after LVAD support. We propose a more sophisticated analysis, of the changes in gene expression between ICM, NICM, and nonfailing hearts. Identifying the shared and distinct genes involved in the development of heart failure of different etiologies may offer insight into cause-specific therapies for heart failure. Second, to demonstrate that molecular signature analysis is feasible in cardiology, we have identified and validated a gene expression profile that differentiates between the two major forms of cardiomyopathy, ICM and NICM.

Table 1.1 Comparing gene discovery and molecular signature analysis

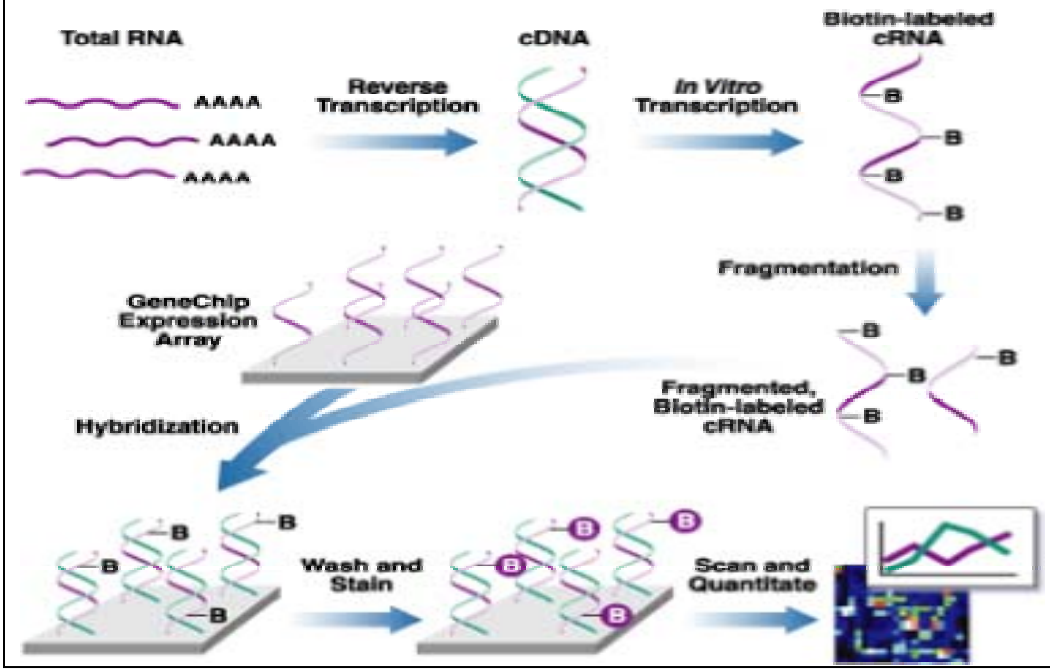
	Gene Discovery	**Molecular Signature Analysis**
Goal of analysis	Elucidate novel genetic pathways and therapeutic targets	Refine diagnosis and treatment
What is identified	Differentially expressed genes between disease states	A pattern of genes that characterizes a clinical parameter: diagnosis, prognosis, or response to therapy
Goal of validation	Confirm levels of gene expression	Assess the predictive accuracy of the molecular signature
Validation strategy	Quantitative PCR Northern blotting RNase protection assays	Apply the molecular signature to independent samples

Figure 1.1 How microarray hybridization works. A. Schematic relating the microarray platform, probe sequences, and scanned, hybridized image. The microarray is a solid support upon which nucleic acid probes corresponding to known gene transcripts are attached in specific locations. B. The many steps involved in microarray analysis. Total RNA is isolated from tissues samples and used to synthesize double-stranded cDNA which is then used as a template to make biotin-labeled cRNA. Fragmented, biotin-labeled cRNA is hybridized to a microarray, containing nucleic acid probes attached to the solid support. After washing, the microarray is scanned. By monitoring the amount of label associated with each probe location, it is possible to infer the abundance of each mRNA species represented.

A


GeneChip Probe Array
Hybridized Probe Cell
Single stranded, labeled RNA target
Oligonucleotide probe
24µm
1.28cm
Millions of copies of a specific oligonucleotide probe
>200,000 different complementary probes
Image of Hybridized Probe Array

B


Total RNA
AAAA
AAAA
AAAA
Reverse Transcription
cDNA
In Vitro Transcription
Biotin-labeled cRNA
B
Fragmentation
GeneChip Expression Array
Fragmented, Biotin-labeled cRNA
Hybridization
Wash and Stain
Scan and Quantitate

Chapter 2

Gene expression analysis of ischemic and nonischemic cardiomyopathy: Shared and distinct genes in the development of heart failure*

*Adapted from Kittleson MM, Minhas KM, Irizarry RA, Ye SQ, Edness G, Breton E, Conte JV, Tomaselli G, Garcia JGN, Hare JM. Gene expression analysis of ischemic and nonischemic cardiomyopathy: Shared and distinct genes in the development of heart failure. *Physiol Genomics* 2005; 21(3):299-307. With permission from the American Physiological Society.

Introduction

Dilated cardiomyopathy is a common cause of congestive heart failure, the leading cause of cardiovascular morbidity and mortality in the United States.[1] Dilated cardiomyopathy can be initiated by a variety of factors, such as ischemia, pressure or volume overload, myocardial inflammation or infiltration, and inherited mutations.[72] A prevailing hypothesis is that despite the varied inciting mechanisms that initiate the heart failure syndrome, there is a final common pathway that drives heart failure progression.[73] Because of this, there is limited research into specific molecular events that are unique to the underlying process. This issue is especially relevant in the two major forms of cardiomyopathy, nonischemic (NICM) and ischemic (ICM), While NICM and ICM have similar presentations,[74] they are characterized by different pathophysiology, prognosis, and response to therapy,[24-27;29;33] and understanding their different pathophysiologic mechanisms is essential in guiding future therapies.

Microarray technology, with the ability to simultaneously assess mRNA levels of tens of thousands of genes, offers a novel approach to compare and contrast the myocardial transcriptome of NICM and ICM. Although previous studies have examined changes in gene expression in failing versus nonfailing (NF) hearts,[41;42;47-49] they have focused only on NICM. The goal of this study was to simultaneously examine the differences in transcriptomes between NICM and ICM and NF hearts to establish a set of shared and unique genes differentially expressed in the two major causes of heart failure. The current analysis offers insight into both disease-specific pathogenesis and therapeutics. Furthermore, an understanding of the distinctions with potential pathophysiologic underpinnings between these two conditions supports and complements

ongoing biomarker development efforts to differentiate heart failure of different etiologies.[75]

Methods

Patient population

The study sample comprised 31 end-stage cardiomyopathy and 6 NF hearts. Myocardial tissue from end-stage cardiomyopathy patients was obtained at the time of left ventricular assist device (LVAD) placement or cardiac transplantation from two institutions: 1) Johns Hopkins Hospital in Baltimore, Maryland (n= 24 NICM and ICM samples and 6 NF samples) and 2) University of Minnesota in Minneapolis, Minnesota (n= 7 NICM samples). Samples from the latter institution were collected and prepared independently,[52] and gene expression data files were kindly provided.

Discarded myocardial tissue from the left ventricular free wall or apex obtained during surgery was immediately frozen in liquid nitrogen and stored at -80° C. The dissecting pathologist selectively excluded areas of visible fibrosis from the portion stored for analysis. Because myocardial tissue obtained at LVAD placement and unused donor hearts are considered discarded tissue, we obtained an exemption from the Johns Hopkins Institution Review Board for sample collection and medical chart abstraction without written informed consent.

Sample preparation

ICM was defined as evidence of myocardial infarction on histology of the explanted heart. In addition, all patients with ICM exhibited severe coronary artery disease (>75% stenosis of the left anterior descending artery and at least one other

epicardial coronary artery) and/or a documented history of a myocardial infarction.[74;76] NICM patients had no history of myocardial infarction, revascularization, or coronary artery disease and had all been diagnosed with idiopathic cardiomyopathy.

Microarray hybridization

Myocardial RNA was isolated from frozen biopsy samples using the Trizol reagent and Qiagen RNeasy columns. Double-stranded cDNA was synthesized from 5 μg RNA using the SuperScript Choice system (Invitrogen Corp, Carlsbad, CA). Each double-stranded cDNA was subsequently used as a template to make biotin-labeled cRNA and 15 μg of fragmented, biotin-labeled cRNA from each sample was hybridized to an Affymetrix U133A microarray (Affymetrix, Santa Clara, California). Affymetrix chip processing was performed at the Hopkins Program for Genomic Applications core facility. The U133A microarray allows detection of 21,722 transcripts (15,713 full length transcripts, 4,534 non-expressed sequence tags (ESTs) and 1,475 ESTs). The quality of array hybridization was assessed by the 3' to 5' probe signal ratio of glyceraldehyde phosphate dehydrogenase (GAPDH) and actin. Our samples had a ratio of 1-1.2, indicating acceptable RNA preparation.

Data normalization

We used the robust multi-array analysis (RMA) algorithm[77;78] to pre-process the Affymetrix probe set data into gene expression levels for all 37 samples (the 30 samples prepared at our institution as described above and the 7 samples prepared at an outside institution[52]). The gene expression data files are accessible through the NCBI Gene Expression Omnibus (GEO) database (accession numbers for series GSE1869; http://www.ncbi.nlm.nih.gov/geo/).

Differential gene expression

We identified differentially expressed genes in two comparisons: 1) NICM versus NF hearts and 2) ICM versus NF hearts. Statistically significant changes in gene expression were identified using Significance Analysis of Microarrays (SAM).[56] SAM identifies genes with statistically significant changes in expression by identifying a set of gene-specific statistics (similar to the t-test) and a corresponding false discovery rate (FDR; similar to a p-value adjusted for multiple comparisons). We identified genes with a FDR of < 5% (corresponding to a p value adjusted for multiple comparisons < 0.05) and an absolute fold change of ≥ 2.0. This threshold has been used in other similar studies[47] and may maximize specificity.[79]

The differentially expressed genes were classified by the Gene Ontology Consortium system (www.geneontology.org) and were visualized by hierarchical clustering and heat mapping[80] using Euclidean distance with complete linkage using software obtained at www.bioconductor.org.

Validation

Levels of transcript normalized to GAPDH (a constitutively expressed gene) were compared between NICM and NF samples and between ICM and NF samples to confirm the up- or down-regulation of differentially regulated transcripts. RNA was available from 4 NF, 5 ICM, and 10 NICM samples. The RNA was treated with DNaseI to remove contaminating genomic DNA and subsequently used to synthesize cDNA. Primers were designed using Primer Express 2.0 software. Each sample was run on a GeneAmp 7900 Sequence Detection System (PE Applied Biosystems) and analyzed using SDS software (Applied Biosystems). For each gene of interest, a standard curve was generated using

serial dilutions of a control cDNA. The quantity of gene transcript in unknown samples was estimated using this standard curve, with GAPDH as a normalizer. SYBR green reagent (Applied Biosystems) served as a reporter throughout all experiments.

Results

Clinical specimens

Subjects with end-stage ICM (n= 10) or NICM (n= 21) exhibited severely reduced ejection fraction, left ventricular dilation, elevated pulmonary arterial and wedge pressures, and reduced cardiac index (Table 2.1). Subjects with ICM were older, all male, more often on angiotensin-converting enzyme inhibitors, and less often on intravenous inotropic therapy. Compared with no-LVAD patients, pre-LVAD patients had lower ejection fraction, higher pulmonary capillary wedge pressure, and lower cardiac index. The NF hearts (n = 6) were from unused cardiac transplant donors. The unused donor subjects were younger (median age 42 years with interquartile range 24-50 years), predominantly male, and information on echocardiographic and hemodynamic parameters and medications was not available.

Differential gene expression: NICM versus NF and ICM versus NF

There were 257 genes differentially expressed between NICM and NF samples and 72 genes differentially expressed between ICM and NF samples with a false discovery rate of $< 5\%$ and an absolute fold change of ≥ 2.0. Of the differentially expressed genes, only 41 were common to both NICM-NF and ICM-NF comparisons.

Differentially expressed genes common to both NICM-NF and ICM-NF comparisons

The majority of the 41 shared genes fell into functional classes of cell growth and maintenance and signal transduction (Figure 2.1). The majority of the genes were up-regulated in NICM and ICM hearts compared with NF hearts, and for all 41 shared genes, fold changes were remarkably similar in direction and magnitude between NICM-NF and ICM-NF comparisons (Table 2.2). Notably, genes implicated in the fetal gene program were differentially expressed. As the ventricle fails, there is a change in the ventricular gene expression pattern from the normal adult pattern to that normally observed only during fetal life, and these genes are known as the fetal gene program.[81]

Genes involved in the fetal gene induction included down-regulation of alpha myosin heavy chain polypeptide 6[82] and up-regulation of atrionatriuretic peptide receptor C in NICM and ICM relative to NF hearts.[83] In the cell growth and maintenance class, there was up-regulation of hemoglobin alpha and beta chains. There was also up-regulation of genes involved in signal transduction, including endothelin receptor type A. In addition, there was significant regulation of genes encoding components of the cytoskeleton (up-regulation collagen type 21 alpha and down-regulation ficolin), and the extracellular matrix (up-regulation of asporin).

Differentially expressed genes unique to the NICM-NF comparison

Of the 216 genes that were uniquely differentially expressed in NICM samples, the majority fell into metabolism, cell growth and maintenance, signal transduction, and binding (Figure 2.1 and Table 2.3). All genes were up-regulated in NICM hearts except one: a zinc transporter which was down-regulated 2-fold. The genes involved in metabolism included angiotensin I-converting enzyme 2 (ACE2) and genes involved in

fatty acid and cholesterol metabolism (acyl-CoA synthetase long-chain family member 3 and oxysterol binding protein-like 8). In cell growth and maintenance, up-regulated genes included cyclin-dependent kinase inhibitor 1B and delta sleep inducing peptide, a vagal-potentiating peptide with influences on cardiac rhythm.[84] Genes involved in signaling pathways were up-regulated, included signal transducer and activator of transcription 1 and 4, members of the JAK/STAT signaling pathway, as well as receptors for leptin, growth hormone, transforming growth factor beta, and platelet-derived growth factor. Several genes implicated in inflammation and the immune response showed increased expression in NICM samples, including interleukin 27, an MHC molecule, and a component of the complement pathway, H factor 1. There were also several genes related to cell adhesion, apoptosis, and development.

Differentially expressed genes unique to the ICM-NF comparison

The 31 genes uniquely differentially expressed between NF and ICM samples mainly belonged to functional classes of cell growth and maintenance, catalytic activity, and signal transduction (Figure 2.1 and Table 2.4). They also included genes implicated in the fetal gene induction, including up-regulation of natriuretic peptide precursor B, atrial natriuretic factor, and an embryonic atrial myosin light chain polypeptide.[72]

Differentially expressed genes and functional categories

To assess whether the difference in functional categories noted in the NICM-NF and ICM-NF comparisons was solely a function of their representation on the microarray platform, we compared the proportion of differentially expressed genes in the different functional categories with their proportional representation on the microarray platform.

As shown in Figure 2.1, the majority of genes on the array (over 50%) belong to functional classes of binding and metabolism; a moderate number of genes (15-40%) are in the classes of catalytic activity, cell growth/maintenance, development, nucleus, signal transduction, and transcription; and few genes (less than 10%) belong to classes of apoptosis, cell adhesion, cytoskeleton, and inflammatory response (the combined percentages total over 100% since genes can belong to more than one functional category). This pattern does not match that of our data ($p < 0.001$ in a χ^2 test). This suggests that the differences in functional categories identified between NICM-NF and ICM-NF comparisons were not solely a function of their representation on the microarray.

Clustering

The heat maps with clustering algorithms for the two comparisons, ICM-NF and NICM-NF, are shown in Figure 2.2. The NF samples formed a distinct cluster from the ICM samples. For the NICM-NF comparison, there were two dominant subclusters. One subcluster contained only NICM samples obtained from patients at the time of LVAD implantation (NICM/pre-LVAD). The other subcluster contained two subgroups: 1) predominantly NF samples and 2) the remaining portion of NICM samples, which were all obtained from patients who did not have an LVAD prior to cardiac transplantation (NICM/no-LVAD). Thus, there was a clear discrimination among the NICM samples: 1) those from patients who required LVADs prior to cardiac transplantation and 2) those from patients who survived to cardiac transplantation without LVAD support.

To determine the specificity of the profiles, we also created a heat map with clustering algorithm for all 288 genes that were differentially expressed in at least one of the two comparisons (Figure 2.3). Samples formed three distinct etiology clusters, but

this was likely due to the presence of shared differentially expressed genes. To confirm the specificity of the differentially expressed genes, we created two additional heat maps with clustering (Figure 2.4): first, NF and ICM samples using only those genes identified as differentially expressed between NF and NICM samples, and second, NF and NICM samples using only those genes identified as differentially expressed between NF and ICM samples. If, as we assumed, the genes uniquely identified as differentially expressed in ICM relative to NF hearts were truly unique to the ICM-NF comparison, then a heat map of these genes in NICM and NF hearts should demonstrate no clustering by etiology. The same should be true for NICM genes in ICM hearts. This was the case: as expected, in both heat maps, the samples did not cluster by etiology, indicating that the unique differentially expressed genes were specific to the given comparison.

Validation

We selected 16 genes of potential biologic interest and validated the microarray findings in NICM, ICM, and NF hearts using QPCR. As shown in Figure 2.5, QPCR confirmed 27 of the 32 microarray predictions with regard to fold change. Of the 5 that did not agree on fold change, 3 were nonsignificantly changed in both comparisons (the leptin receptor in ICM, serine proteinase inhibitor, clade E, member 1 in NICM, and the acyl-CoA synthetase long-chain family member 3 in ICM), leaving only 2 clear disagreements: S100 calcium binding protein A8 was significantly down-regulated by QPCR but nonsignificantly up-regulated by microarray and lumican was significantly up-regulated in ICM by microarray and non-significantly downregulated by QPCR.

Discussion

The principal finding of this investigation is that cardiomyopathies of different etiologies exhibit both shared and distinct changes in gene expression compared with NF hearts. Remarkably, of more than 22,000 transcripts present on the Affymetrix microarray platform, only 288 genes (1-2%) are differentially expressed in NICM and ICM relative to NF hearts, and 41 of these genes are common to both comparisons with comparable fold changes. This suggests that there are both shared and distinct mechanisms that contribute to the development of heart failure of different etiologies, and a better understanding of these distinctions supports ongoing efforts to develop cause-specific therapies specifically targeted at NICM and ICM.[85]

The current study is unique for a number of reasons. First, we have studied 37 samples, which is large relative to gene expression studies in cardiomyopathy to date.[41;42;47-53;55] There are no accepted means of calculating sample size and power in microarray experiments, but because our study examines a larger number of samples than prior studies, we have increased power to detect significant changes in gene expression. Furthermore, we have the added advantage of uniformity among samples: all NICM samples were from individuals with idiopathic cardiomyopathy, and their clinical characteristics were similar.

The second unique feature of this study is that we have compared not only NICM and NF hearts, as in many previous studies,[41;42;48;49] but extended this analysis to compare the differential gene expression of NICM and ICM relative to NF hearts. This offers further insight into the mechanisms involved in the development of heart failure of varying etiologies. Many genes are shared between NICM and ICM relative to NF hearts,

and this is consistent with clinical experience: the presentations and standard treatment for cardiomyopathy of both etiologies is similar.[1] However, despite similar presentations and therapies, NICM and ICM are distinct diseases; patients with ICM have decreased survival compared with their NICM counterparts,[29;33] and respond differently to therapies.[24-27] Thus, an understanding of the distinctions between the two conditions at the level of gene expression may guide future efforts to design etiology-based therapies.

The predominance of metabolism genes in NICM hearts suggests that the derangements involved in the genesis and maintenance of NICM may be metabolic in nature. This is supported by an early trial of beta-blockers in heart failure which demonstrated a greater mortality benefit in NICM than ICM.[5] Beta-blockers improve myocardial efficiency by shifting myocardial metabolism from free fatty acids to glucose. The increase in fatty acid metabolism genes specifically in NICM in our analysis would explain why beta-blockers may be particularly beneficial in NICM. Furthermore, our results suggest that future etiology-specific therapies in NICM could target metabolic pathways, including those of fatty acid or cholesterol synthesis. One particularly relevant example is ranolazine. This investigational compound shifts myocardial cells from fatty acid to glucose metabolism and is currently being investigated as a treatment for myocardial ischemia.[86] Based on our results, this drug may also be helpful in patients with NICM.

In ICM, on the other hand, our results suggest that abnormalities in catalytic activity may predominate. Notably, our analysis demonstrated the down-regulation of a specific serine proteinase inhibitor which has an anti-ischemic protective effect in pigs subject to experimentally-induced myocardial ischemia.[87] Thus, its down-regulation in

ICM samples may be a maladaptive response to ischemic injury. Given our results, it may be possible that repletion of such an enzyme could be beneficial in patients with ICM.

Our work agrees to an extent with the findings of a similar analysis of differential gene expression by Steenman et al.,[47] in which pooled samples of NICM and ICM were compared to one NF sample, and 95 differentially expressed genes were identified. When compared to our list of 288 genes, we found 8 genes in common (Table 2.5). There are a number of reasons why our results differed. The prior study had only one NF heart, and it was from a patient with cystic fibrosis. This heart is likely very different, not only in age, but also in hemodynamic parameters, from a heart from an unused cardiac transplant donor. In addition, we used different statistical algorithms for normalization and identification of differentially expressed genes. We normalized with RMA, which has been shown to offer better detection of differentially expressed genes than Affymetrix's default preprocessing algorithm.[78] We identified differentially expressed genes with Significance Analysis of Microarrays, which has been validated in a number of studies[56;66;67;88] and may be more accurate than other commonly used methods for identifying differentially expressed genes, such as t-tests.[89] In addition, our analysis may have more external validity because we studied more samples (37 versus 7 patients) with individually hybridized, as opposed to pooled, data. Individual hybridization may be more accurate than pooling because it allows the estimation of the within-group variance for each gene.[90]

Some of the genes shown to be differentially expressed in our study have been previously identified as differentially expressed in studies of NF versus NICM hearts,

with remarkably similar fold changes between studies (Table 2.5). Commonly identified genes include those involved in the fetal gene program,[72] including natriuretic peptide precursor B, atrial natriuretic factor, cardiac muscle myosin heavy chain, and atrial alkali myosin light chain. The majority of genes are up-regulated in NICM and ICM hearts versus NF hearts, and this has also been noted in prior studies.[41;42;47-49] This is likely due to biologic differences, since prior studies all used different methods to normalize data and identify differentially expressed genes. Furthermore, since the expression of many of these genes was confirmed with quantitative PCR in these prior studies, this offers indirect further confirmation of the validity of our differentially expressed genes. This highlights the critical point in microarray analysis used for gene discovery: the results should be considered hypothesis-generating and the gene expression should be confirmed with other quantitative techniques, such as quantitative PCR.[57]

Through quantitative PCR, we confirmed the expression of 27 of the 32 comparisons with 16 genes of interest in heart failure. Of greatest interest are the novel genes from our analysis, including ACE2 and a member of the tumor necrosis factor receptor superfamily (TNFRSF11B, also known as osteoprotegerin). We show that in subjects with end-stage cardiomyopathy, ACE2 is significantly up-regulated in NICM but not ICM. ACE2 is expressed predominantly in vascular endothelial cells of the heart and kidney, and ACE and ACE2 have different biochemical activities. Angiotensin I is converted to angiotensin I–9 (with nine amino acids) by ACE2 but is converted to angiotensin II, which has eight amino acids, by ACE. Whereas angiotensin II is a potent blood-vessel constrictor, angiotensin I–9 has no known effect on blood vessels but can be converted by ACE to a shorter peptide, angiotensin I–7, which is a blood-vessel dilator.[91]

The up-regulation of ACE2 in NICM but not ICM cannot be ascribed to the increased prescription of ACE inhibitors in ICM subjects because unlike ACE, ACE2 is insensitive to inhibition by ACE inhibitors.[92] Thus, our results offer insight into a possible new etiology-specific therapeutic target in heart failure.

Another novel finding of interest is the significant down-regulation of a member of the tumor necrosis factor receptor subfamily, TNFRSF11B in both NICM and ICM. Levels of tumor necrosis factor (TNF) are elevated in chronic heart failure[93] and increasing levels of TNF have been correlated with disease severity.[94] However, in clinical trials, soluble TNF-alpha antagonists did not reduce mortality or heart failure hospitalizations.[95;96] One might speculate that this lack of benefit may relate to the down-regulation of the TNF receptor in chronic heart failure.

The results of the unsupervised hierarchical clustering algorithm suggest that patients with NICM who do not undergo LVAD implantation resemble their NF counterparts more than patients with NICM patients who require an LVAD prior to cardiac transplantation. An examination of their baseline characteristics confirms this: NICM-LVAD patients are a sicker subset, with higher pulmonary capillary wedge pressure and increased need for intravenous inotropes, two known markers of poor prognosis in chronic heart failure patients.[97;98] While there are documented changes in gene expression between hearts before and after LVAD support,[51-53;55] there is no evidence that differential gene expression exists between end-stage cardiomyopathy samples obtained before LVAD placement and at the time of cardiac transplantation or between patients with different clinical presentations. Because this result was obtained with an unsupervised clustering algorithm, it is free of bias of predefined categories.[63]

While is it possible that the differences were due, in part, to the use of 7 NICM-LVAD samples from an outside institution, this is less likely because the outside institution samples themselves did not form a distinct cluster. This unanticipated difference between end-stage NICM patients could offer insight into the differential gene expression of different stages of heart failure. This requires further study, and lends credence to the notion that gene expression can be correlated with clinically relevant parameters in heart failure patients to aid in determining prognosis and response to therapy.

Although the analysis of gene expression using oligonucleotide microarrays is a powerful technique, limitations warrant mention. Not all genes are represented on the Affymetrix U133A arrays used in this study, and therefore the knowledge that can be acquired from these experiments remains incomplete. In addition, an unused donor heart is not the same as a normal heart, because circumstances causing to a donor heart being ineligible for cardiac transplantation, such as infection or prolonged hypotension, can also affect gene expression. In fact, one study suggested that the differential gene expression identified between NICM and NF hearts may have been due to age and gender differences rather than differences in ventricular function.[42] However, normal, age- and sex-matched hearts are impossible to obtain, and other researchers have used comparable unused donor hearts in their experiments.[41;42;48;49]

Another limitation of this study is that microarray analysis is essentially hypothesis generating. However, in the tradition of such studies in the microarray literature,[41-43;47-49;51-53] this is a hypothesis-generating analysis with biologic validation of select genes confirmed by QPCR. We have followed the practice of other studies in the field, and extended the analysis to include more samples with different etiologies of heart

failure and a careful comparison with the results of prior studies (Table 2.5), which is unprecedented in the literature thus far. For this reason, we believe that these analyses, while mainly hypothesis-generating, do have significant value and should be made available to other individuals interested in microarray analysis of ICM and NICM.

In conclusion, we offer a novel addition to the analysis of differential gene expression between failing and nonfailing hearts by providing new insight into the genetic pathways involved in the genesis of cardiomyopathy of different etiologies. This analysis could provide a basis for future studies of cause-specific therapies in the complex management of heart failure patients.

Table 2.1 Clinical characteristics of the study subjects used to identify differentially expressed genes between ischemic and nonischemic cardiomyopathy and nonfailing hearts

	Ischemic		Nonischemic	
	No LVAD* (n = 7)	Pre-LVAD† (n = 3)	No LVAD* (n = 8)	Pre-LVAD* (n = 13)
Age, y	54 (49 – 60)	60 (59 – 60)	51 (48 – 53)	46 (37 – 52) §
Male	100%	100%	86%	62%
Ejection fraction, %	20 (15 -25)	17.5 (10 – 25)	17. 5(7.5 – 27.5)	15 (12.5 – 20)
LVIDd, cm	6.8 (6.7 – 7.6)	6.5 (6 – 7)	8.4 (7.5 – 9.3)	7.3 (6.8 – 8.1)
PCWP, mm Hg	15 (12 – 23)	30 (30 – 32)	13.5 (13 – 14)	27 (21 – 31) ‡
Cardiac index, $L \cdot min^{1} \cdot m^{2}$	2.4 (2.3 -2 .4)	1.4 (1.3 – 1.5) ‡	2.4 (1.9 – 2.8)	1.5 (1.3 – 1.6)
β-adrenergic antagonists	71%	67%	38%	36%
ACE inhibitors or ARBs	100%	100%	88%	55%
Diuretics	100%	100%	100%	64%
Inotropic therapy[a]	100%	33%	13%	73% ‡

Values are median (25^{th} and 75^{th} percentiles)*, median (range)†, or percentages. ACE is angiotensin-converting enzyme, ARB is angiotensin receptor blocker, LVAD is left ventricular assist device; LVIDd is left ventricular end-diastolic diameter, PCWP is pulmonary capillary wedge pressure.

‡ $p < 0.05$ for difference between no-LVAD and pre-LVAD groups.

§ $p < 0.05$ for difference between ischemic and nonischemic cardiomyopathy.

[a]Includes dopamine, dobutamine, and milrinone.

Table 2.2 Differentially expressed genes (n=41) shared between the ischemic-cardiomyopathy-versus-nonfailing heart and nonischemic-cardiomyopathy-versus-nonfailing-heart comparison

Gene symbol	**Gene name**	**ICM-NF**		**NICM-NF**	
		Fold change*	**FDR**	**Fold change***	**FDR**
Cell growth/maintenance					
HBA2	hemoglobin, alpha 2	4.3	0.50	2.7	0.18
HSAGL2	human alpha-globin gene	3.5	0.50	2.4	0.18
HBB	hemoglobin, beta	3.4	0.50	2.6	0.18
HBA2	hemoglobin, alpha 2	3.4	0.50	2.2	0.18
HBA1	hemoglobin, alpha 1	3.3	0.50	2.1	0.18
AF059180	mutant beta-globin gene	3.0	0.50	2.4	0.18
HBB	hemoglobin, beta	3.0	0.50	2.6	0.18
DUT	dUTP pyrophosphatase	2.2	0.50	2.2	0.18
RARRES1	retinoic acid receptor responder 1	-3.0	0.90	-2.2	0.52
Signal transduction					
PIK3R1	phosphoinositide-3-kinase, reg subunit, polypeptide 1	3.1	0.50	2.3	0.18
NPR3	atrionatriuretic peptide receptor C	3.1	0.50	2.5	0.18
CBLB	Cas-Br-M ectropic retroviral transforming sequence b	2.3	0.50	2.3	0.18
EDNRA	endothelin receptor type A	2.1	2.76	2.1	0.52
DKFZp564I1922	Adlican	2.0	1.28	2.4	0.18
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	-2.7	1.69	-2.0	1.18

SCYA2	small inducible cytokine A2	-3.5	0.90	-2.9	0.18
Metabolism					
EIF1AY	eukaryotic translation initiation factor 1A	2.2	0.50	2.2	0.60
KIAA0669	KIAA0669 gene product	2.2	0.50	3.2	0.18
SFPQ	splicing factor proline/glutamine rich	2.1	0.50	2.0	0.18
Nucleus					
PHLDA1	pleckstrin homology-like domain, family A, member 1	3.5	0.50	5.1	0.18
PHLDA1	pleckstrin homology-like domain, family A, member 1	3.3	0.50	4.9	0.18
ANP32E	acidic nuclear phosphoprotein 32 family, member E	2.0	0.50	2.7	0.18
Cell adhesion/cell communication					
COL21A1	collagen, type XXI, alpha 1	2.3	0.50	2.3	0.18
FCN3	ficolin 3	-3.2	0.90	-2.6	0.18
Catalytic activity					
DBY	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide	2.4	0.50	2.7	0.52
AGXT2L1	alanine-glyoxylate aminotransferase 2-like 1	-2.5	0.90	-2.4	0.18
Binding					
PEPP2	phosphoinositol 3-phosphate-binding protein-2	2.2	0.50	2.4	0.18
QKI	homolog of mouse quaking QKI	2.1	0.50	2.0	0.18
Other					
MYT1	myelin transcription factor 1	2.0	0.90	2.4	0.18
ASPN	asporin (LRR class 1)	2.1	0.50	3.3	0.18
MYH6	myosin, heavy polypeptide 6, cardiac muscle, alpha	-2.5	0.50	-3.7	0.18
AF000381	folate binding protein mRNA, partial cds.	3.7	0.50	3.0	0.18
TPR	translocated promoter region	2.5	0.50	2.2	0.18

none	Homo sapiens, clone IMAGE:4182947, mRNA	2.3	0.50	3.0	0.18
none	Homo sapiens, clone IMAGE:4182947, mRNA	2.3	0.50	3.1	0.18
none	Homo sapiens, clone IMAGE:3611719, mRNA	2.2	0.50	2.1	0.18
none	Homo sapiens cDNA FLJ11918 fis	2.2	0.50	2.8	0.18
P311	similar to Neuronal protein 3.1	2.1	0.90	2.4	0.18
none	Human clone 23589 mRNA sequence	2.1	0.90	2.6	0.18
HMG2	high-mobility group (nonhistone chromosomal) protein 2	2.1	0.50	3.1	0.18
SERPINA3	serine (or cysteine) proteinase inhibitor,clade A, mem 3	-2.5	0.50	-2.0	0.18

*Fold change described the mean gene expression for ischemic and nonischemic samples relative to nonfailing samples. FDR is false discovery rate, analogous to a p value (as a percentage) adjusted for multiple comparisons.

NICM-NF denotes comparison between nonfailing hearts and nonischemic cardiomyopathy samples

ICM-NF denotes comparison between nonfailing hearts and ischemic cardiomyopathy samples

Table 2.3 Differentially expressed genes (n = 216) unique to the nonischemic-cardiomyopathy-versus-nonfailing-heart comparison*

Gene symbol	Gene name	Fold change*	FDR
Metabolism			
FACL3	acyl-CoA synthetase long-chain family member 3	2.8	0.18
HNRPH3	heterogeneous nuclear ribonucleoprotein H3	2.7	0.18
FLJ22222	hypothetical protein FLJ22222 (protein metabolism)	2.6	0.18
OSBPL8	oxysterol binding protein-like 8	2.6	0.18
ACE2	angiotensin I converting enzyme 2	2.6	0.68
VDU1	pVHL-interacting deubiquitinating enzyme 1	2.4	0.18
LIPA	lipase A, lysosomal acid, cholesterol esterase	2.4	0.18
MGEA5	meningioma expressed antigen 5	2.4	0.18
FLJ12552	hypothetical protein FLJ12552	2.4	0.18
CPE	carboxypeptidase E	2.4	0.18
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	2.4	0.18
SFRS7	splicing factor, arginine/serine-rich 7	2.3	0.18
YT521	splicing factor YT521-B, KIAA1966	2.3	0.18
SMARCA2	SWI/SNF related, matrix assoc, actin dep reg of chromatin	2.3	0.18
GLS	Glutaminase	2.3	0.18
CTSB	cathepsin B	2.3	0.18
RNASE4	ribonuclease, RNase A family, 4	2.3	0.18
DPYD	dihydropyrimidine dehydrogenase	2.3	0.18
GATM	glycine amidinotransferase	2.3	0.18
HSP105B	heat shock 105kD	2.2	0.18
GATM	glycine amidinotransferase	2.2	0.18
PIGK	phosphatidylinositol glycan, class K	2.2	0.18
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	2.2	0.18
BACE	beta-site APP-cleaving enzyme	2.2	0.18
NBS1	Nijmegen breakage syndrome 1	2.1	0.18
LUC7A	cisplatin resistance-associated overexpressed protein	2.1	0.18
UBE1C	ubiquitin-activating enzyme E1C	2.1	0.18
GCH1	GTP cyclohydrolase 1	2.0	1.2
C15orf15	chromosome 15 open reading frame 15	2.0	0.18
FBXO3	F-box only protein 3	2.0	0.18

ODC1	ornithine decarboxylase 1	2.0	0.18
B3GALT3	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 3	2.0	0.52
SEPP1	selenoprotein P, plasma, 1	2.0	0.18
SLC39A8	solute carrier family 39, member 8	-2.0	0.18
Cell growth/maintenance			
NAP1L3	nucleosome assembly protein 1-like 3	3.1	0.18
ARID4B	AT rich interactive domain 4B	2.5	0.18
CDKN1B	cyclin-dependent kinase inhibitor 1B	2.5	0.18
RNPC2	RNA-binding region containing 2	2.4	0.18
DUSP6	dual specificity phosphatase 6	2.3	0.18
NAP1L1	nucleosome assembly protein 1-like 1	2.3	0.18
DENR	density-regulated protein	2.3	0.18
CENTB2	centaurin, beta 2	2.2	0.18
TOB1	transducer of ERBB2, 1	2.2	0.18
SEC23A	Sec23 homolog A	2.2	0.18
SNAP23	synaptosomal-associated protein, 23kD	2.2	0.18
ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	2.2	0.18
SEC24B	SEC24 related gene family, member B	2.2	0.18
CGI-142	hepatoma-derived growth factor 2	2.2	0.18
BMI1	B lymphoma Mo-MLV insertion region	2.2	0.18
ABCA8	ATP-binding cassette, sub-family A, member 8	2.1	0.18
BC003689	high-mobility group nucleosomal binding domain 2	2.1	0.18
GAPCENA	rab6 GTPase activating protein	2.1	0.18
PURA	purine-rich element binding protein A	2.1	0.18
NUP153	nucleoporin 153kD	2.1	0.18
PLSCR4	phospholipid scramblase 4	2.1	0.18
NAB1	NGFI-A binding protein 1	2.1	0.18
TRIM33	tripartite motif-containing 33	2.1	0.18
DSIPI	delta sleep inducing peptide, immunoreactor	2.1	0.18
CTBP2	C-terminal binding protein 2	2.1	0.18
JJAZ1	joined to JAZF1	2.1	0.18
ZFHX1B	zinc finger homeobox 1b	2.0	0.18
ZNF161	zinc finger protein 161	2.0	0.18
SERP1	stress-associated endoplasmic reticulum protein 1	2.0	0.18

Signal transduction			
APM1	adipocyte, C1Q and collagen domain containing	3.5	0.52
SH3BGRL	SH3 domain binding glutamic acid-rich protein like	3.1	0.18
ARHI	ras homolog gene family, member I	2.7	0.18
ERBB2IP	erbb2 interacting protein	2.6	0.18
P23	unactive progesterone receptor, 23 kD	2.5	0.68
SH3BP5	SH3-domain binding protein 5	2.4	0.18
GHR	growth hormone receptor	2.4	0.18
APP	amyloid beta precursor protein	2.4	0.18
STAT1	signal transducer and activator of transcription 1, 91kD	2.4	0.18
TCF7L2	transcription factor 7-like 2	2.4	0.18
PDE4B	phosphodiesterase 4B, cAMP-specific	2.3	0.52
STC1	stanniocalcin 1	2.3	0.52
TGFBR3	transforming growth factor, beta receptor III	2.3	0.18
LEPR	leptin receptor	2.3	0.18
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	2.2	0.18
PENK	proenkephalin	2.1	0.18
ATP6IP2	ATPase, H+ transporting, lysosomal interacting protein 2	2.1	0.18
IGFBP3	insulin-like growth factor binding protein 3	2.1	0.18
ROCK1	Rho-associated, coiled-coil containing protein kinase 1	2.1	0.18
OGN	osteoglycin	2.1	0.18
LIM	LIM protein	2.1	0.18
AKAP11	A kinase anchor protein 11	2.1	0.18
TCF7L2	transcription factor 7-like 2	2.1	0.18
PDGFC	platelet derived growth factor C	2.1	0.18
NCOA2	nuclear receptor coactivator 2	2.0	0.18
Binding			
KIAA0882	KIAA0882 protein	2.3	0.18
TRIM22	tripartite motif-containing 22	2.3	0.18
KIAA0993	WD repeat and FYVE domain containing 3	2.2	0.18
BC017580	stress 70 protein chaperone, microsome-associated, 60kDa	2.2	0.18
SE70-2	cutaneous T-cell lymphoma tumor antigen se70-2	2.2	0.18
EPS15	epidermal growth factor receptor pathway substrate 15	2.2	0.18
MYCBP2	MYC binding protein 2 , KIAA0916	2.2	0.18
MATR3	matrin 3	2.2	0.18

PLAGL1	pleiomorphic adenoma gene-like 1	2.1	0.18
KIAA0853	KIAA0853 protein	2.1	0.18
ZZZ3	zinc finger, ZZ domain containing 3 , DKFZP564I052	2.1	0.18
MATR3	matrin 3	2.1	0.18
CRI1	CREBBP/EP300 inhibitory protein 1	2.1	0.18
FMR1	fragile X mental retardation 1	3.3	0.18
Transcription factors			
YY1	YY1 transcription factor	2.4	0.18
SP3	Sp3 transcription factor	2.4	0.18
RBBP1	retinoblastoma binding protein 1	2.3	1.2
NR2F2	nuclear receptor subfamily 2, group F, member 2	2.3	0.18
SOX4	SRY (sex determining region Y)-box 4	2.3	0.18
STAT4	signal transducer and activator of transcription 4	2.1	0.18
ELK3	ELK3, ETS-domain protein	2.1	0.18
Inflammation/immune response			
HF1	H factor 1 (complement)	2.5	0.18
NR3C1	nuclear receptor subfamily 3, group C, member 1	2.1	0.18
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	2.3	0.18
IL27	interleukin 27	2.0	0.52
Development			
LUM	lumican	2.8	0.18
FRZB	frizzled-related protein	2.1	0.18
DIXDC1	DIX domain containing 1, KIAA1735	2.0	0.18
ATP2C1	ATPase, Ca++ transporting, type 2C, member 1	2.0	0.18
OSF-2	periostin, osteoblast specific factor	3.0	0.18
Cell adhesion			
PNN	pinin, desmosome associated protein	2.3	0.68
LAMB1	laminin, beta 1	2.3	0.18
DPT	dermatopontin	2.2	0.18
Catalytic activity			
HNMT	histamine N-methyltransferase	2.2	0.18
HS2ST1	heparan sulfate 2-O-sulfotransferase 1	2.1	0.18
PHKB	phosphorylase kinase, beta	2.1	0.18
DKFZP586A052 2	DKFZP586A0522 protein	2.0	0.18

Apoptosis			
BNIP3L	BCL2/adenovirus E1B 19kD interacting protein 3-like	2.2	0.18
SPF30	survival motor neuron domain containing 1	2.2	0.18
TIA1	TIA1 cytotoxic granule-associated RNA binding protein	2.1	0.18
BCL2	B-cell CLL/lymphoma 2	2.0	0.18
Cytoskeleton			
DMD	dystrophin	2.2	0.18
ADD3	adducin 3	2.1	0.18
KLHL2	kelch-like 2, Mayven	2.0	0.18
Other			
KTN1	kinectin 1 (kinesin receptor)	2.7	0.18
C8orf2	chromosome 8 open reading frame 2	2.2	0.18
GCC2	GRIP and coiled-coil domain containing 2 , KIAA0336	2.0	0.18
AF054589	I-mfa domain-containing protein	2.2	0.18
EFA6R	ADP-ribosylation factor guanine nucleotide factor 6	3.0	0.18
AF130089	Homo sapiens clone FLB9440 PRO2550 mRNA, complete cds.	2.9	0.18
AF130082	Homo sapiens clone FLC1492 PRO3121 mRNA, complete cds.	2.9	0.18
AF070641	Homo sapiens clone 24421 mRNA sequence	2.7	0.18
AF271775	Homo sapiens DC49 mRNA, complete cds.	2.7	0.18
CG005	phosphonoformate immuno-associated protein 5	2.6	0.18
KIDINS220	likely homolog of rat kinase D-interacting substance of 220 kDa	2.6	0.18
ALEX3	ALEX3 protein	2.5	0.18
KIAA0680	chromosome 6 open reading frame 56	2.5	0.18
FLJ11273	hypothetical protein FLJ11273	2.4	0.18
UBQLN2	ubiquilin 2	2.4	0.18
DICER1	Dicer1, Dcr-1 homolog (Drosophila)	2.4	0.18
RYBP	RING1 and YY1 binding protein	2.4	0.18
TEB4	similar to S. cerevisiae SSM4	2.3	0.18
IPW	imprinted in Prader-Willi syndrome	2.3	0.52
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta	2.3	0.18
SP329	likely ortholog of mouse modulator of KLF7 activity	2.3	0.18
SDCCAG1	serologically defined colon cancer antigen 1	2.3	0.52
MARCKS	myristoylated alanine-rich protein kinase C substrate	2.3	0.18

AK027252	Homo sapiens clone 23664 and 23905 mRNA sequence	2.3	0.18
EPS8	epidermal growth factor receptor pathway substrate 8	2.3	0.18
AK055910	Homo sapiens cDNA FLJ31348 fis, clone MESAN2000026	2.2	0.18
KIAA0143	KIAA0143 protein	2.2	0.18
AK025583	Homo sapiens cDNA clone	2.2	0.18
KIAA0914	family with sequence similarity 13, member A1	2.2	0.18
STAG2	stromal antigen 2	2.2	0.18
AL136139	Contains 3' part of the gene for enhancer of filamentation (HEF1)	2.2	0.18
M55536	Human glucose transporter pseudogene	2.2	0.18
AASDHPPT	aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	2.2	0.18
PTN	pleiotrophin	2.2	0.18
MGC4276	HESB like domain containing 2	2.2	0.18
LOC51110	lactamase, beta 2	2.2	0.18
GATA6	GATA binding protein 6	2.2	0.18
AK021980	Homo sapiens cDNA FLJ11918 fis, clone HEMBB1000272	2.2	0.18
AK025216	Homo sapiens cDNA: FLJ21563 fis, clone COL06445	2.2	0.18
none	chromosome 6 open reading frame 111: DKFZp564B0769	2.2	0.18
AK021980	Homo sapiens cDNA FLJ11918 fis, clone HEMBB1000272	2.2	0.18
13CDNA73	hypothetical protein CG003	2.1	0.18
GASP	G protein-coupled receptor-associated sorting protein , KIAA0443	2.1	0.18
PSIP2	PC4 and SFRS1 interacting protein 2	2.1	0.18
ARL5	ADP-ribosylation factor-like 5	2.1	0.18
KIAA0582	KIAA0582 protein	2.1	0.18
FLJ23018	hypothetical protein FLJ23018	2.1	0.18
none	hypothetical protein DKFZp761K1423	2.1	0.52
STAG2	stromal antigen 2	2.1	0.18
SACS	spastic ataxia of Charlevoix-Saguenay (sacsin)	2.1	0.18
AW190289	ESTs	2.1	0.18
KIAA1109	KIAA1109 protein	2.1	0.18
KPNB3	karyopherin (importin) beta 3	2.1	0.18
TTC3	tetratricopeptide repeat domain 3	2.1	0.18
AK055600	Homo sapiens mRNA; cDNA DKFZp434G012	2.1	0.18
HHL	expressed in hematopoietic cells, heart, liver , KIAA0471	2.1	0.18

RCP	Rab coupling protein	2.1	0.18
FLJ22104	hypothetical protein FLJ22104	2.1	0.18
BTN3A3	butyrophilin, subfamily 3, member A3	2.1	0.18
BCMP1	transmembrane 4 superfamily member 10	2.1	0.18
AV712064	EST: Homo sapiens cDNA: DCAAUD05, 5'end, human dendrites	2.1	0.18
RNF38	ring finger protein 38 , FLJ21343	2.1	0.18
AL049998	Homo sapiens mRNA; cDNA DKFZp564L222	2.1	0.18
HS696H22	Human DNA sequence from clone RP4-696H22	2.1	0.18
BC007568	Homo sapiens, clone IMAGE:3028427, mRNA, partial cds	2.1	0.18
DICER1	Dicer1, Dcr-1 homolog (Drosophila)	2.1	0.18
HS21C048	Homo sapiens chromosome 21 segment HS21C048.	2.1	0.18
XPO1	exportin 1 (CRM1 homolog, yeast)	2.0	0.18
ALEX1	ALEX1 protein	2.0	0.18
KIAA0372	KIAA0372 gene product	2.0	0.18
DC8	DKFZP566O1646 protein	2.0	0.60
FAM3C	family with sequence similarity 3, member C, GS3786	2.0	0.18
AL713745	Homo sapiens mRNA; cDNA DKFZp761J0523	2.0	0.18
TTC3	tetratricopeptide repeat domain 3	2.0	0.18
TTC3	tetratricopeptide repeat domain 3	2.0	0.18
UNC84A	unc-84 homolog A (C. elegans), KIAA0810	2.0	0.18
OAZIN	ornithine decarboxylase antizyme inhibitor	2.0	0.18
ZNF292	ZNF292 zinc finger protein 292 , KIAA0530	2.0	0.18
PJA2	praja 2, RING-H2 motif containing , KIAA0438	2.0	0.18
HNRPA3	heterogeneous nuclear ribonucleoprotein A3	2.0	0.18
HS73M23	ESTs	2.0	0.18
RECQL	RecQ protein-like (DNA helicase Q1-like)	2.0	0.18
DR1	down-regulator of transcription 1, TBP-binding (negative cofactor 2)	2.2	0.18
AL049437	Homo sapiens mRNA; cDNA DKFZp586E1120	2.2	0.18

*Fold change described the mean gene expression for ischemic and nonischemic samples relative to nonfailing samples. FDR is false discovery rate, analogous to a p value (as a percentage) adjusted for multiple comparisons.

Table 2.4 Differentially expressed genes (n = 31) unique to the ischemic-cardiomyopathy-versus-nonfailing heart comparison*

Gene symbol	Gene name	Fold change*	FDR
Cell growth/maintenance			
RPS4Y	ribosomal protein S4, Y-linked	2.4	0.50
ALS2CR3	amyotrophic lateral sclerosis 2 chromosome region, candidate 3	2.3	0.50
KPNB2	karyopherin beta 2	2.1	0.50
SLC16A7	solute carrier family 16, member 7	2.1	0.50
ZNF145	zinc finger protein 145	2.1	1.1
Catalytic activity			
SERPINB1†	serine (or cysteine) proteinase inhibitor, clade B, member 1	-2.2	0.90
SERPINB1†	serine (or cysteine) proteinase inhibitor, clade B, member 1	-2.2	2.4
ATP1B3	ATPase, Na+/K+ transporting, beta 3 polypeptide	-2.3	0.90
SERPINE1	serine (or cysteine) proteinase inhibitor,clade E, member 1	-2.3	3.0
Signal transduction			
NPPB	natriuretic peptide precursor B	4.4	2.8
HSCDDANF	Human cardiodilatin-atrial natriuretic factor	2.3	3.8
PBEF	pre-B-cell colony-enhancing factor	-2.1	3.7
Transcription factors			
ATF3	activating transcription factor 3	-2.6	3.0
SMAP31	homeodomain-only protein	-3.3	0.90
Inflammation/immune response			
PTX3	pentaxin-related gene, rapidly induced by IL-1 beta	-2.2	3.0
S100A8	S100 calcium binding protein A8 (calgranulin A)	-2.7	0.90
Development			
ARIH2	ariadne homolog 2	2.0	0.50
DLK1	delta-like 1 homolog	2.0	2.9
Metabolism			
PLA2G2A	phospholipase A2, group IIA	-3.4	0.90
Cytoskeleton			
MYL4	myosin, light polypeptide 4, alkali; atrial, embryonic	2.4	2.0

Other			
AF116676	EMBL: Homo sapiens PRO1957 mRNA, complete cds.	2.3	2.4
TXNIP	thioredoxin interacting protein	2.3	0.50
SYNPO2L	synaptopodin 2-like	2.1	0.50
FLJ11539	hypothetical protein FLJ11539	2.1	0.50
FLJ10159	hypothetical protein FLJ10159	2.0	0.50
none	Homo sapiens cDNA FLJ11918 fis, clone HEMBB1000272	2.0	0.50
DKFZP434F0318	hypothetical protein DKFZp434F0318	2.0	2.0
none	Homo sapiens cDNA: FLJ22179 fis, clone HRC00920	2.0	0.50
CD163	CD163 antigen	-2.0	0.90
none	Homo sapiens cDNA FLJ30298 fis, clone BRACE2003172	-2.0	0.90
none	Homo sapiens cDNA: FLJ21545 fis, clone COL06195	-3.0	0.90

*Fold change described the mean gene expression for ischemic and nonischemic samples relative to nonfailing samples. FDR is false discovery rate, analogous to a p value (as a percentage) adjusted for multiple comparisons.

†There are two entries for this gene product because it was identified as differentially expressed with two unique Affymetrix accession numbers.

Table 2.5 Differentially expressed genes between ischemic and nonischemic cardiomyopathy and nonfailing hearts common to previously published reports

	Fold change					
Gene symbol	**Our study NICM-NF**	**Our study ICM-NF**	**Tan [48]**	**Barrans [41]**	**Yung [49]**	**Steenman [47]**
PHLDA1	5.1	3.5			5.43	
PIK3R1	2.3	3.1				2.73
TPR	2.2	2.5			2.02	
COL21A1	2.3	2.3			3.52	
EIF1AY	2.2	2.2		1.78		
MYH6	-3.7	-2.5			-5.3	-1.36
FCN3	-2.6	-3.2			-7.7	
NPPB		4.4	3.3			7.24
MYL4		2.4		2.01		3.79
HSCDDANF		2.3	4.2	19.15		4.83
ZNF145		2.1				2.33
ATP1B3		-2.3			-2.7	
PLA2G2A		-3.4	-5.1			
FMR1	3.3				2.06	
SH3BGRL	3.1					1.20
OSF-2	3.0		12	1.96		
LUM	2.8		3.8			
HNRPH3	2.7				1.83	
HF1	2.5			1.23		
CDKN1B	2.5				2.03	
PDE4B	2.3			2.41		
PTN	2.2				3.29	
ATP6IP2	2.1			1.19		
GAPCENA	2.1				1.74	
TIA1	2.1			2.14		
PLAGL1	2.1				2.2	
NR3C1	2.1				1.72	
DSIPI	2.1					1.29
FBXO3	2.0				1.59	
ODC1	2.0				2.52	

Gene symbols correspond to gene products as noted in Tables 2.2-2.4.

Figure 2.1 Percent of known genes in each functional category that were significantly regulated in both nonischemic (NICM) and ischemic (ICM) cardiomyopathy compared to nonfailing (NF) hearts (black bars), unique to NICM hearts (light gray bars), unique to ICM hearts (white bars), and the representation of these functional categories on the array (dark grey bars). There is no correlation with the representation of genes on the array and distribution of genes in the comparisons. APO is apoptosis, BIN is binding, CAT is catalytic activity, CEL is cell adhesion, CGM is cell growth/maintenance, CYT is cytoskeleton, DEV is development, INF is inflammatory response, MET is metabolism, NUC is nucleus, SIG is signal transduction, and TRA is transcription.

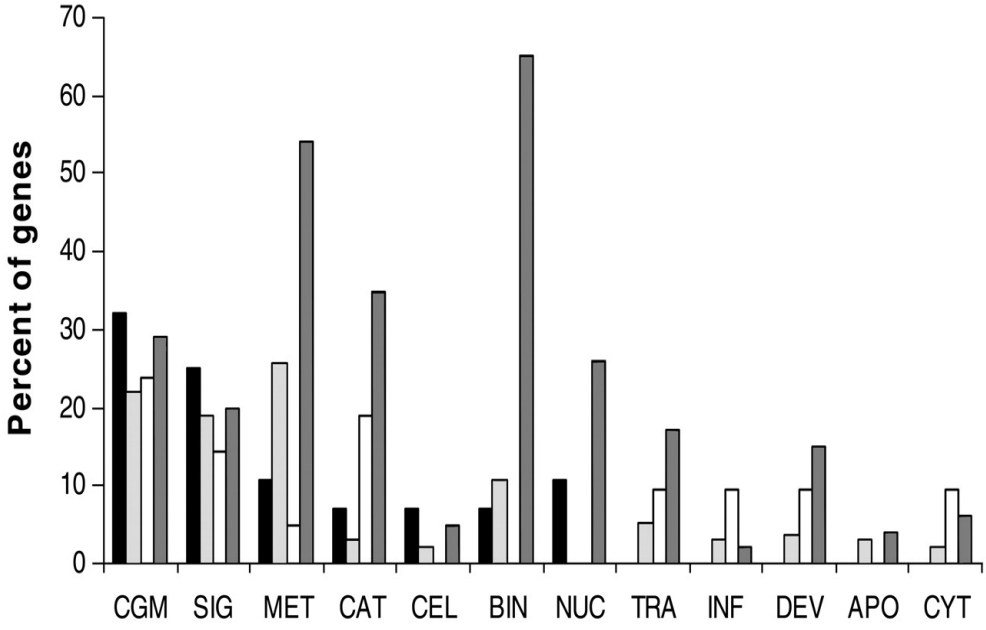


Figure 2.2 Hierarchical clustering of genes significantly regulated in ischemic (ICM; A) and nonischemic cardiomyopathy (NICM; B) compared to nonfailing (NF) hearts considered separately. Each row represents a gene and each column represents a sample. Sample prefixes "T" denotes end samples from patients at the time of cardiac transplantation without left ventricular assist devices (no-LVAD); "LC" denotes samples obtained from patients at the time of LVAD placement (pre-LVAD), and "N" denotes nonfailing samples. The suffix "i" denotes ICM samples. The suffix "ni" denotes NICM samples. The color in each cell reflects the level of expression of the corresponding gene in the corresponding sample, relative to its mean level of expression in the entire set of samples. Expression levels greater than the mean are shaded in blue, and those below the mean are shaded in red. Circled samples denote the predominant etiology clusters and samples labeled with an arrow fall outside of their appropriate cluster. A. NF versus ICM. The no- and pre-LVAD samples do not form distinct clusters. B. NF versus NICM. The no- and pre-LVAD samples form distinct clusters, as indicated.

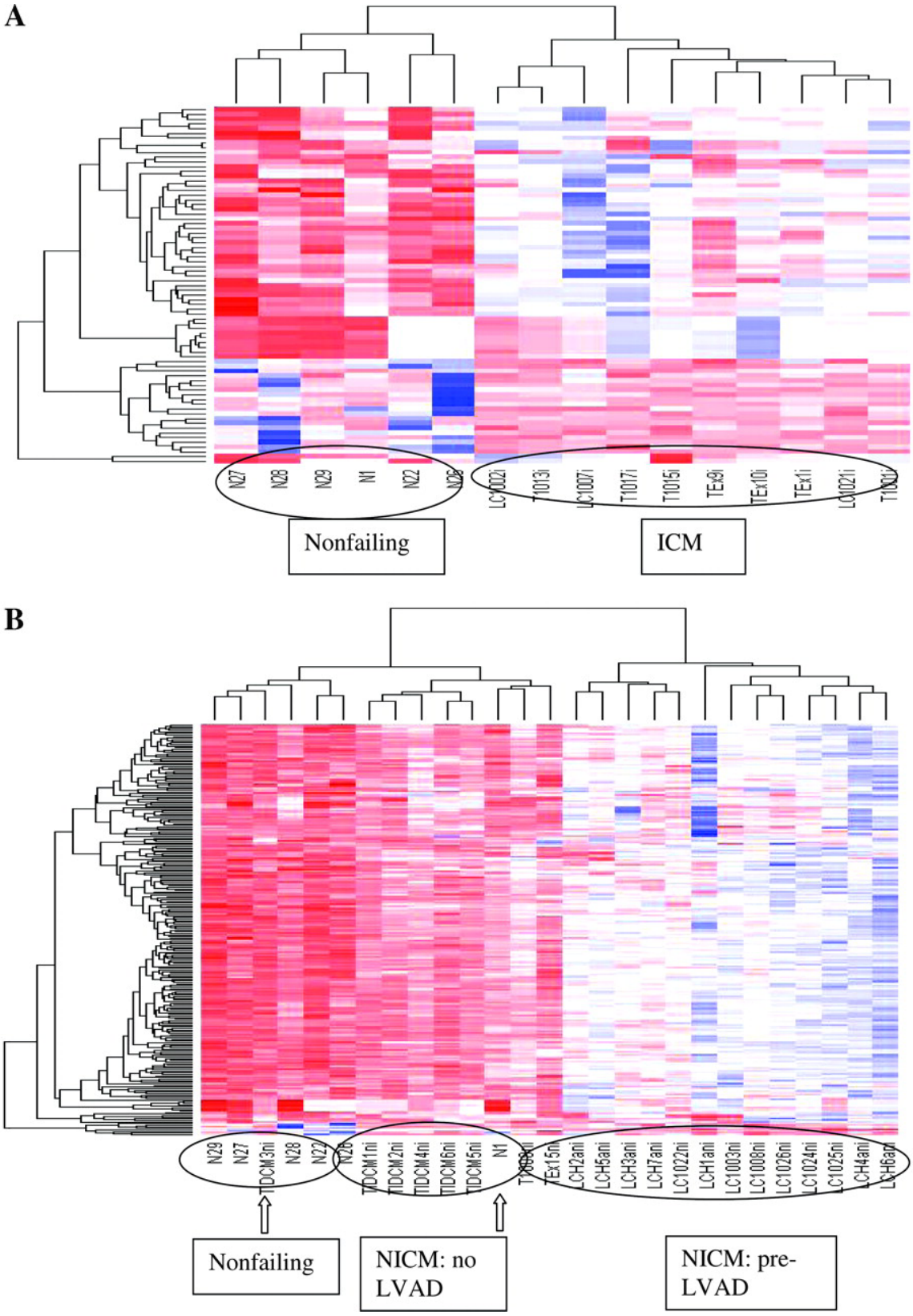

A
N27
N28
N29
N1
N22
N26
LC1002i
T1013i
LC1007i
T1017i
T1015i
TEx9i
TEx10i
TEx1i
LC1021i
T1001i
Nonfailing
ICM
B
N29
N27
TIDCM3ni
N28
N22
N26
TIDCM1ni
TIDCM2ni
TIDCM4ni
TIDCM6ni
TIDCM5ni
N1
TEx15ni
LCH2ani
LCH5ani
LCH3ani
LCH7ani
LC1022ni
LCH1ani
LC1003ni
LC1008ni
LC1026ni
LC1024ni
LC1025ni
LCH4ani
LCH6ani
Nonfailing
NICM: no LVAD
NICM: pre-LVAD

Figure 2.3 Hierarchical clustering of genes significantly regulated in nonischemic cardiomyopathy (NICM) and ischemic cardiomyopathy (ICM) compared to nonfailing (NF) hearts considered together. All 288 genes that were differentially expressed in either the NICM-NF or ICM-NF comparison are included. Each row represents a gene and each column represents a sample. Sample prefixes "T" denotes end samples from patients at the time of cardiac transplantation without left ventricular assist devices (LVADs); "LC" denotes samples obtained from patients at the time of LVAD placement (pre-LVAD), and "N" denotes nonfailing samples. The suffix "i" denotes ICM samples. The suffix "ni" denotes NICM samples. The color in each cell reflects the level of expression of the corresponding gene in the corresponding sample, relative to its mean level of expression in the entire set of samples. Expression levels greater than the mean are shaded in blue, and those below the mean are shaded in red. Circled samples denote the predominant etiology clusters.

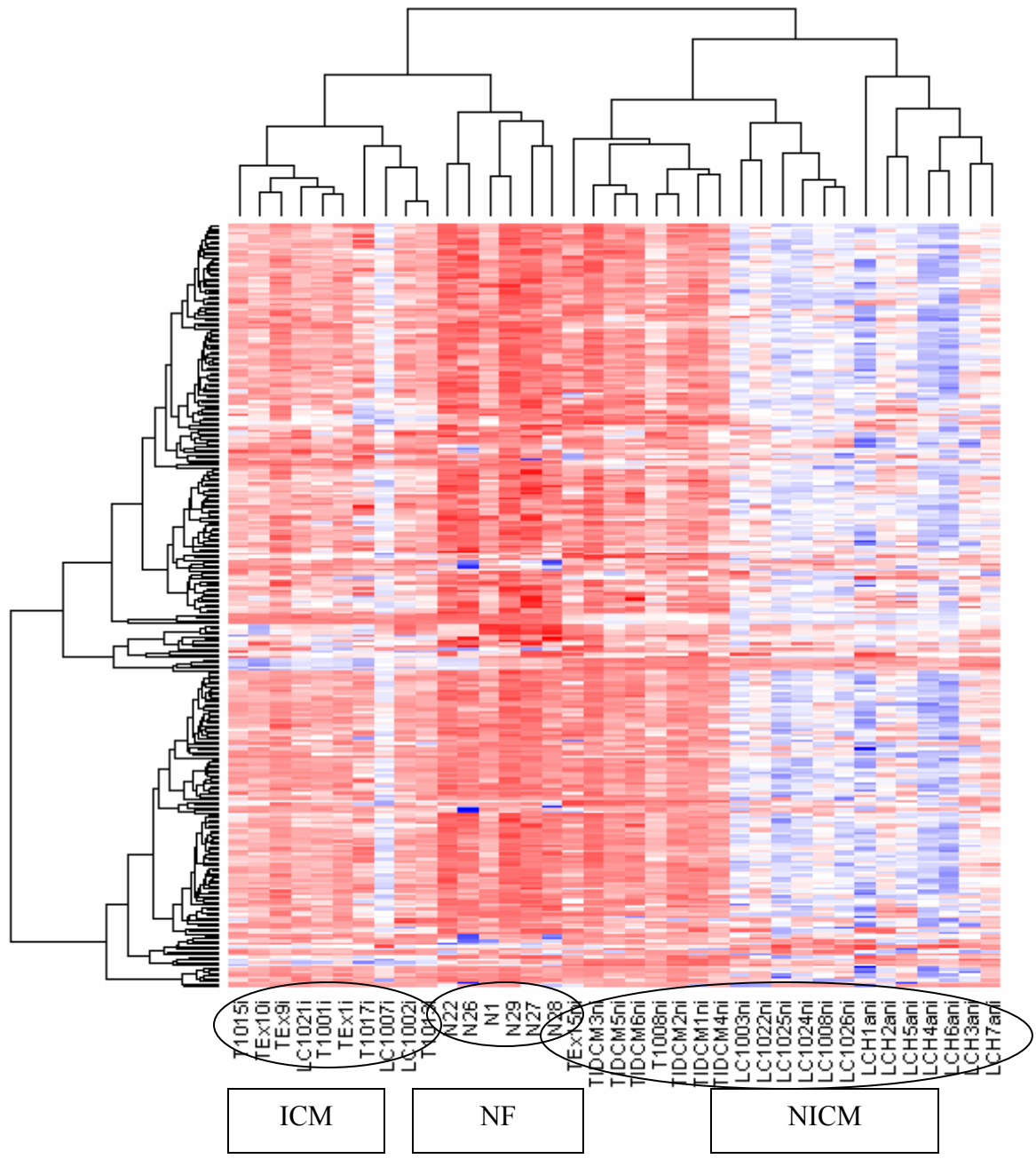
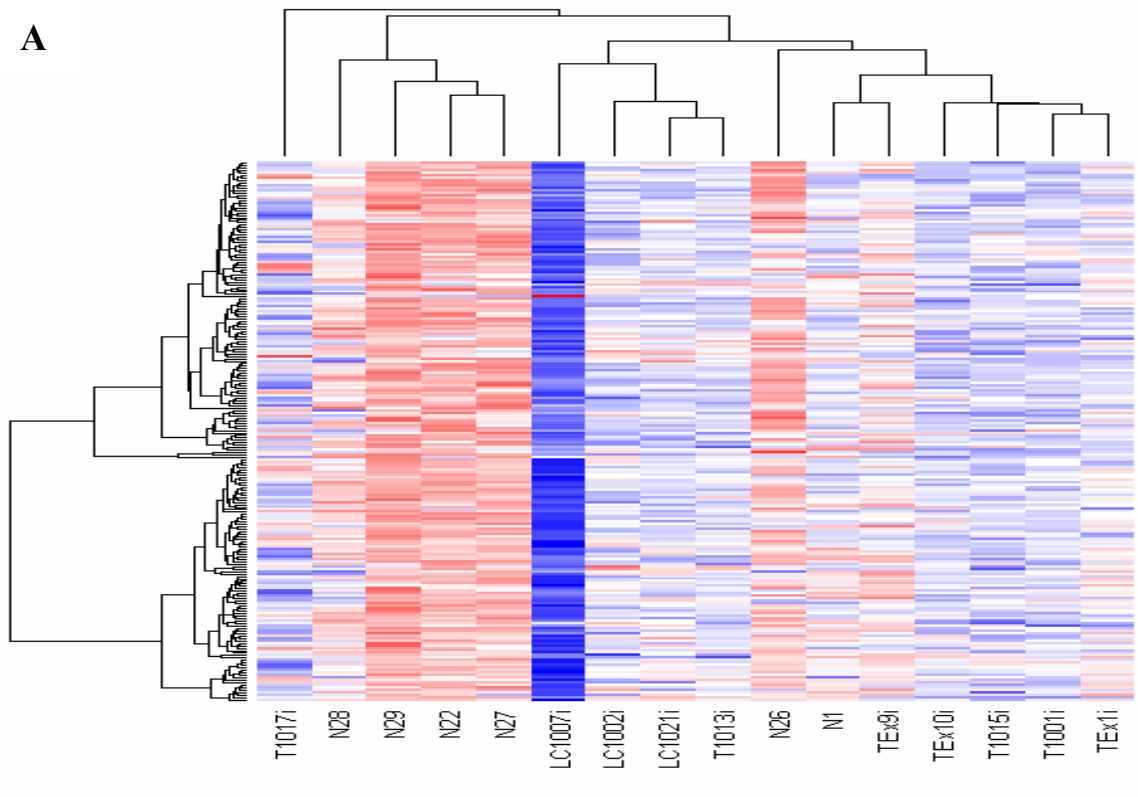

T1015i
TEx10i
TEx9i
LC1021i
T1001i
TEx1i
T1017i
LC1007i
LC1002i
T1013i
N22
N26
N1
N29
N27
N28
TEx15ni
TIDCM3ni
TIDCM5ni
TIDCM6ni
T1008ni
TIDCM2ni
TIDCM1ni
TIDCM4ni
LC1003ni
LC1022ni
LC1025ni
LC1024ni
LC1008ni
LC1026ni
LCH1ani
LCH2ani
LCH5ani
LCH4ani
LCH6ani
LCH3ani
LCH7ani
ICM
NF
NICM

Figure 2.4 Hierarchical clustering of genes significantly regulated in nonischemic cardiomyopathy tested in ischemic cardiomyopathy and nonfailing hearts (A) and regulated in ischemic cardiomyopathy tested in nonischemic cardiomyopathy and nonfailing hearts (B). Each row represents a gene and each column represents a sample. Sample prefixes "T" denotes end samples from patients at the time of cardiac transplantation without left ventricular assist devices (LVADs); "LC" denotes samples obtained from patients at the time of LVAD placement (pre-LVAD), and "N" denotes nonfailing samples. The suffix "i" denotes ischemic cardiomyopathy samples. The suffix "ni" denotes nonischemic cardiomyopathy samples. A. Nonfailing versus ischemic cardiomyopathy using those genes identified as differentially expressed in the nonfailing-nonischemic comparison. The samples do not form distinct etiology clusters. B. Nonfailing versus nonischemic cardiomyopathy using only those genes identified as differentially expressed in the nonfailing-ischemic comparison. The samples do not form distinct etiology clusters.


A
T1017i
N28
N29
N22
N27
LC1007i
LC1002i
LC1021i
T1013i
N26
N1
TEx9i
TEx10i
T1015i
T1001i
TEx1i

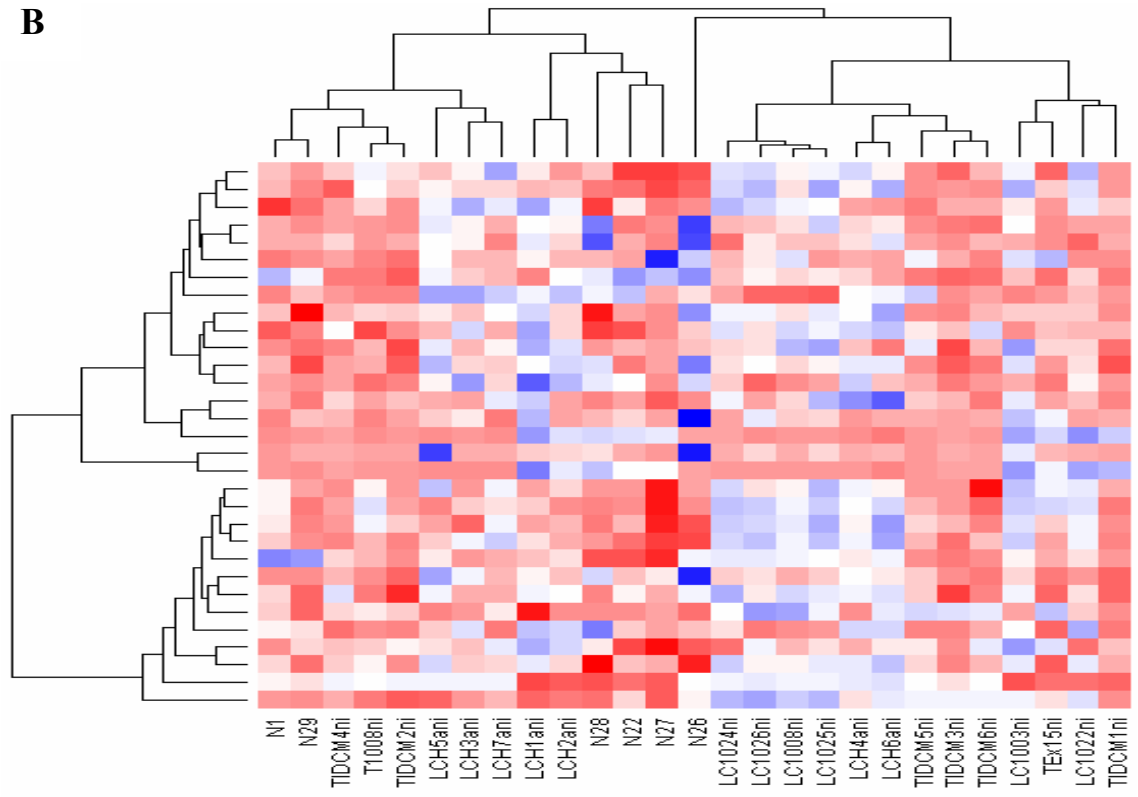
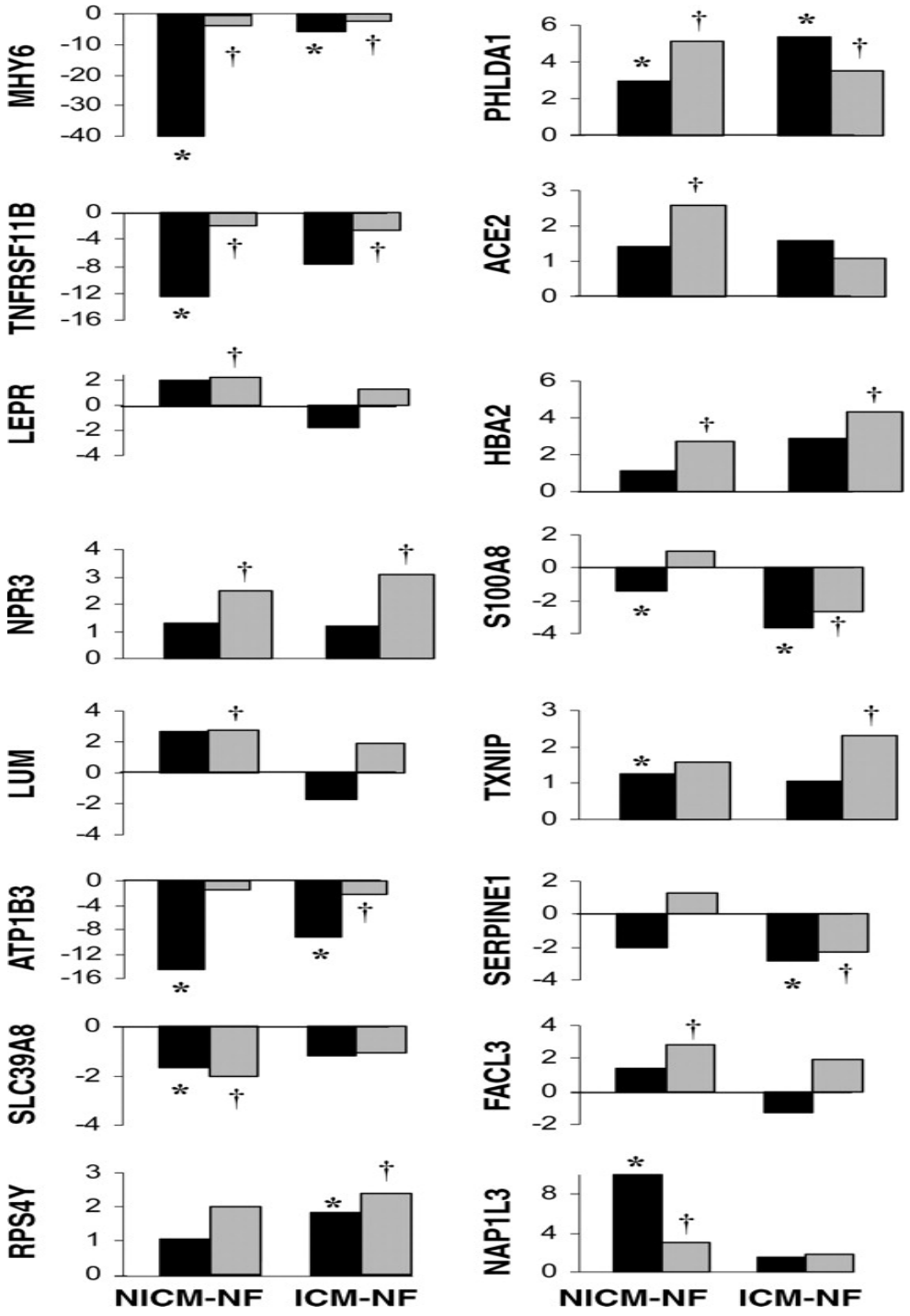

B
N1
N29
TIDCM4ni
T1008ni
TIDCM2ni
LCH5ani
LCH3ani
LCH7ani
LCH1ani
LCH2ani
N28
N22
N27
N26
LC1024ni
LC1026ni
LC1008ni
LC1025ni
LCH4ani
LCH6ani
TIDCM5ni
TIDCM3ni
TIDCM6ni
LC1003ni
TEx15ni
LC1022ni
TIDCM1ni

Figure 2.5 Independent assessment of gene expression levels by quantitative PCR for select genes differentially expressed in ischemic and nonischemic cardiomyopathy relative to nonfailing hearts. Fold change in expression in nonischemic (NICM) and ischemic (ICM) hearts compared with nonfailing (NF) hearts according to QPCR (black bars) and microarrays (gray bars). ACE2, angiotensin-converting enzyme 2; ATP1B3, ATPase, Na+/K+ transporting, beta 3 polypeptide; FACL3, acyl-CoA synthetase long-chain family member 3; HBA2, hemoglobin A2; LEPR, leptin receptor; LUM, lumican; MYH6, myosin heavy chain 6; NAP1L3, nucleosome assembly protein 1-like 3; NPR3, atrionatriuretic peptide receptor C; PHLDA1, pleckstrin homology-like domain family A member 1; RPS4Y, ribosomal protein S4, Y-linked; S100A8, S100 calcium binding protein A8; SERPINE1, serine (or cysteine) proteinase inhibitor,clade E, member 1; SLC39A8, solute carrier family 39, member 8; TNFRSF11B, tumor necrosis factor receptor superfamily member 11b; TXNIP, thioredoxin interaction protein. * $P < 0.05$ compared with NF hearts by Wilcoxon rank sum test. † $P < 0.05$ by Significance Analysis of Microarrays.


MHY6
PHLDA1
TNFRSF11B
ACE2
LEPR
HBA2
NPR3
S100A8
LUM
TXNIP
ATP1B3
SERPINE1
SLC39A8
FACL3
RPS4Y
NAP1L3
NICM-NF
ICM-NF
NICM-NF
ICM-NF

Chapter 3

Identification of a Gene Expression Profile that Differentiates between Ischemic and Nonischemic Cardiomyopathy*

*Adapted from Kittleson MM, Ye SQ, Irizarry RA, Minhas KM, Edness G, Conte JV, Parmigiani G, Miller LW, Chen Y, Hall JL, Garcia JGN, Hare JM. Identification of a Gene Expression Profile that Differentiates between Ischemic and Nonischemic Cardiomyopathy. *Circulation* 2004; 110(22): 3444-51. With permission from Lippincott Williams and Wilkins.

Introduction

Molecular signature analysis through gene expression profiling has the potential to refine diagnostic and prognostic accuracy in a variety of diseases. This technique has enjoyed widespread success in solid and hematological malignancies[62;64-67;99;100] and may soon be employed in clinical trials. While the ability to refine diagnosis and predict patient outcome has tremendous importance in myocardial diseases, the application of molecular signature analysis is in its earliest stages. Small studies offer novel insights into gene expression in failing and nonfailing hearts,[41-43;47-49] dilated and hypertrophic cardiomyopathy,[50] and before and after ventricular assist device placement.[51-53;55] However, gene expression analysis has not yet been used to distinguish clinically relevant cardiovascular disease subtypes. In fact, molecular signature analysis for cardiomyopathy is considered controversial due to the contention that, unlike tumors, there is a final common pathway independent of etiology for the progression of myocardial disease.[73]

The aim of this study was to test the hypothesis that molecular signature analysis using gene expression profiling could discriminate between the two major forms of cardiomyopathy, ischemic (ICM) and nonischemic (NICM). We demonstrate that this methodology is applicable to samples obtained in different institutions and is specific to disease stage. This study establishes proof-of-principle that gene expression signatures have the potential to refine the evaluation and treatment of heart failure patients, where management decisions may vary based on disease etiology.[21;24;25;29;33;101] Our findings strongly support ongoing efforts to incorporate gene expression-based biomarkers in determining prognosis and response to therapy in heart failure.

Materials and methods

Patients

The study comprised 48 samples. Myocardial tissue from different disease stages was obtained: 1) at left ventricular assist device (LVAD) placement or cardiac transplantation (n = 25 "end-stage"); 2) following LVAD support (n = 16; "post-LVAD"); and 3) from prospectively collected newly diagnosed patients at endomyocardial biopsy (n = 7; "biopsy"). Samples were from two institutions: 1) the Johns Hopkins Hospital (n = 34) and 2) the University of Minnesota (n = 14). Samples from the latter institution were collected and prepared independently,[52] and the gene expression data files were kindly provided.

Written informed consent was obtained from all patients undergoing endomyocardial biopsy for sample collection and medical chart abstraction. Myocardial tissue obtained at LVAD placement, following LVAD support, or at cardiac transplantation, however, is considered discarded tissue. Therefore, we obtained an exemption from the Johns Hopkins Institution Review Board for its collection and medical chart abstraction without written informed consent.

ICM was defined as histological evidence of ischemic injury and all ICM patients exhibited severe coronary artery disease (>75% stenosis of the left anterior descending artery and at least one other proximal epicardial artery) and/or a documented history of myocardial infarction.[76] NICM patients had no history of myocardial infarction, revascularization, or coronary artery disease. Newly diagnosed patients were those presenting or referred to the Johns Hopkins Hospital with a new diagnosis of

cardiomyopathy and symptoms for 6 months or less[29] for further diagnostic evaluation, which included endomyocardial biopsy.

Sample collection

For end-stage and post-LVAD samples, discarded myocardial tissue from the left ventricular free wall or apex obtained during surgery was immediately frozen in liquid nitrogen and stored at -80° C. The dissecting pathologist selectively excluded areas of visible fibrosis from the portion stored for analysis.

For biopsy samples, patients referred to the Johns Hopkins Hospital with a newly diagnosed cardiomyopathy underwent endomyocardial biopsy as part of their evaluation. Right heart catheterization was performed from the right internal jugular vein. Endomyocardial biopsy of the distal RV septum was performed with a Mansfield 2.2 mm jaw size bioptome (Boston Scientific Corp., Watertown, MA) under fluoroscopic guidance to ensure proper positioning of the biopsy forceps. A portion of the biopsy, two samples of endomyocardium weighing 2-4 g, were immediately frozen in liquid nitrogen and stored at -80° C.

Microarray hybridization

Myocardial RNA was isolated from frozen end-stage and post-LVAD samples using the Trizol reagent and Qiagen RNeasy columns. For biopsy samples, RNA isolation included additional steps with a microhomogenizer (Fisher Scientific International, Pittsburgh, PA) and an RNAqueous-micro isolation kit (Ambion Inc, Austin, TX), and 50 ng total RNA as a starting material were subjected to two-round amplification using the BioArray RNA Amplification and Labeling kit (Enzo Life Sci., Inc., Farmingdale, NY). For end-stage samples, double-stranded cDNA was synthesized

from 5 μg RNA using the SuperScript Choice system (Invitrogen Corp, Carlsbad, CA). Each double-stranded cDNA was subsequently used as a template to make biotin-labeled cRNA and 15 μg of fragmented, biotin-labeled cRNA from each sample was hybridized to an individual Affymetrix U133A microarray (Affymetrix, Santa Clara, California). Affymetrix chip processing was performed at the Hopkins Program for Genomic Applications core facility. The U133A is an oligonucleotide microarray that allows detection of 21,722 transcripts (15,713 full length, 4,534 non-expressed sequence tags (ESTs), and 1,475 ESTs).

Quality control

All the arrays were subject to quality control according to the parameters established by Affymetrix for assessing sample and array quality.[40] These parameters included measurement of 260/280nm ratios of both total RNA and biotinylated cRNA; visual inspection for the presence of image artifacts; and assessment of average background and noise value, exogenously added prokaryotic hybridization controls, percent present calls, scaling factors, and internal control genes (actin and glyceraldehyde phosphate dehydrogenase (GAPDH)). Only arrays in which all parameters were within acceptable levels were subject to further analysis. Specifically, our samples had a ratio of < 3.0, indicating acceptable RNA preparation and ICM and NICM samples had similar proportions of present calls (45 – 48%).

Data normalization

We used the robust multiarray average (RMA) algorithm[77;78] to normalize the Affymetrix probe set data into gene expression levels for all 48 samples. Both the samples from the Johns Hopkins Hospital and those collected and prepared at the

University of Minnesota[52] were normalized in the same manner. There are four stages to RMA. First, probe-set data from all arrays are simultaneously normalized using quantile normalization, which eliminates systematic differences between GeneChips without significantly altering the relative intensity of probes within a GeneChip. Second, the mean optical background level for each array is estimated, and the intensity for each probe is adjusted to remove this. Third, the normalized, background-corrected data is transformed to the $\log_2$ scale. Finally, a median-polish procedure is used to combine multiple probes into a single measure of expression for each gene on each array. Although we initially normalized the data with Affymetrix's default preprocessing algorithm (MAS 5.0), we found that RMA resulted in classifiers with better predictive power. These results are consistent with a publication showing that RMA provides better detection of differentially expressed genes than MAS 5.0.[78]

Filtering

In order to create the gene expression signature using only genes that were differentially expressed in ICM versus NICM samples, we first analyzed the microarrays with Significance Analysis of Microarrays (SAM).[56] SAM identifies genes with statistically significant changes in expression by identifying a set of gene-specific statistics (similar to the t-test) and a corresponding false discovery rate (FDR; similar to a p value adjusted for multiple comparisons). At a FDR of 10%, there were 3332 differentially expressed genes between ICM and NICM. These 3332 genes were then subject to further analysis.

Prediction analysis

To develop a gene expression signature that distinguished ICM from NICM, we employed Prediction Analysis of Microarrays (PAM)[62] implemented in the R package for statistical computing, and a number of prediction analyses were performed (Figure 3.1). Sixteen end-stage cardiomyopathy samples (6 ICM and 10 NICM) from Johns Hopkins Hospital formed a training set to develop the etiology signature. There were three test sets to validate the signature: 1) the remaining 9 end-stage cardiomyopathy samples, including 7 from the University of Minnesota; 2) 16 post-LVAD samples; and 3) 7 biopsy samples.

Because the accuracy of the signature could differ based on the random division of samples into training and test tests, the above analysis was repeated with 210 random partitions of the samples into a 16-sample training set and 9-sample test set to determine the overall accuracy. Each random partition identified different, overlapping sets of genes, but a 90-gene signature repeatedly minimized the cross-validation error. We applied PAM to the entire set of 25 end-stage samples to identify the 90-gene signature as the representative etiology molecular signature.

The genes in the etiology molecular signature were classified by the Gene Ontology Consortium system (www.geneontology.org) and the signature was visualized by hierarchical clustering and a heat map (www.bioconductor.org) using Euclidean distance with complete linkage.

Statistical analysis

Continuous variables were summarized by the median and quartiles and groups were compared using the Wilcoxon rank sum test. Categorical variables were

summarized by proportions and compared using Fisher's exact test. Sensitivity was defined as the proportion of ICM samples correctly classified by the gene expression signature, and specificity was defined as the proportion of NICM samples correctly classified.

To assess if the accuracy of the etiology molecular signature was affected by baseline clinical characteristics (including age, gender, systolic function, and medication use) as well as differences in etiology, we divided the 25 end-stage cardiomyopathy subjects into multiple two-way strata based on age (≥ or < 50 years), systolic function (≥ or < 15%), and use of intravenous inotropes. We then assessed the representative etiology signature's sensitivity and specificity over these strata by determining the proportion of subjects within each stratum that were correctly classified as having ICM or NICM.

Quantitative PCR

Levels of transcript normalized to GAPDH (a constitutively expressed gene) were compared between ICM and NICM end-stage samples to confirm the up- or down-regulation of select genes in the etiology signature. RNA samples were treated with DNaseI to remove contaminating genomic DNA and subsequently used to synthesize cDNA. Primers were designed using Primer Express 2.0 software. Each sample was run on a GeneAmp 7900 Sequence Detection System (PE Applied Biosystems) and analyzed using SDS software (Applied Biosystems). For each gene of interest, a standard curve was generated using serial dilutions of a control cDNA. The quantity of gene transcript in unknown samples was estimated using this standard curve, with GAPDH as a

normalizer. SYBR green reagent (Applied Biosystems) served as a reporter throughout all experiments.

Results

Patient characteristics

All end-stage patients exhibited severely reduced ejection fraction, left ventricular dilation, elevated pulmonary arterial and wedge pressures, and reduced cardiac index (Table 3.1). Compared with end-stage NICM patients, end-stage and post-LVAD ICM patients were older and all male. In addition, compared with end-stage NICM patients, end-stage ICM patients were all on angiotensin-converting enzyme (ACE) inhibitors and less often on intravenous inotropes. Hemodynamic and remodeling indices were similar between end-stage ICM and NICM patients. Newly diagnosed ICM patients were older than their NICM counterparts with better remodeling indices.

Diagnostic accuracy

We developed an etiology signature using a training set of samples that, when applied to independent end-stage ICM and NICM samples, demonstrated 100% sensitivity and 100% specificity. This perfect accuracy was also achieved in a test set where the majority of samples were from an institution distinct from that used to create the signature.

We assessed whether the etiology signature was affected by disease stage. In post-LVAD samples, the gene expression signature correctly classified all NICM samples (n=13; specificity 100%), but only classified 1 of 3 ICM samples correctly (sensitivity 33%). In biopsy samples from patients with newly diagnosed cardiomyopathy, the signature again correctly classified all NICM samples (n = 4; specificity 100%), but only

classified 1 of 3 ICM samples correctly (sensitivity 33%). The overall accuracy over 210 random partitions of training and test sets was sensitivity 89% (95% CI 75 – 100%) and specificity 89% (95% CI 60 – 100%).

Effect of clinical characteristics

We examined the signature's predictive accuracy in strata based on each clinical characteristic (Table 3.2). All the ICM patients were male and on ACE inhibitors so we could not ascertain if the signature would apply to women with ICM who were not on ACE inhibitors. However, within each stratum, the sensitivity and specificity were similar and all were comparable to the overall sensitivity and specificity.

Characterization of the etiology prediction signature

Over 210 combinations of training and test set samples, the greatest accuracy was achieved with signatures containing 90 genes. A 90-gene expression signature exhibited perfect accuracy 30% of the time. The majority of genes in the representative etiology prediction profile were involved in signal transduction, metabolism, and cell growth/maintenance (Figure 3.2). Most were up-regulated in ICM with an average fold change of 1.9 ± 0.9 (Table 3.3).

In a hierarchical clustering algorithm, 11 of the 16 ICM samples and 30 of the 32 NICM samples formed a distinct cluster (Figure 3.3). Whereas the biopsy samples clustered together, the samples did not cluster by pre- or post-LVAD status or by institution of origin.

Quantitative PCR

Levels of transcript for four genes in the etiology signature were confirmed using quantitative PCR (Figure 3.4). In all four cases, the direction of the fold change by microarray and quantitative PCR were the same.

Discussion

The major new finding of this report is the identification of a gene expression signature that accurately distinguishes ICM and NICM. The signature was applicable to samples from different laboratories and, for NICM, was independent of disease stage. Gene expression signatures have been successfully correlated with etiology and outcome in oncology.[62;64-67;99;100] There is an equal need to refine diagnostic and prognostic techniques in myocardial diseases, but advances have been restricted by limited tissue access. Our findings demonstrate that gene expression signatures can accurately identify etiology in cardiovascular disease, and support ongoing efforts to incorporate gene expression-based biomarkers in determining prognosis and response to therapy.

While the main focus of this study was proof-of-principle, a gene expression signature that distinguishes ICM and NICM could provide a valuable adjunct to diagnostic imaging and metabolic tools. ICM and NICM are distinct diseases; patients with ICM have decreased survival compared to their NICM counterparts[29;33;33] and respond differently to therapies.[21;24;25;101] An etiology molecular signature would offer diagnostic insight, especially in patients with heart failure out of proportion to their coronary artery disease, up to 11% in one observational study.[76]

Although we have demonstrated that end-stage cardiomyopathy can be accurately classified by gene expression, a more relevant signature would focus on newly diagnosed patients. Therefore, we also tested the end-stage etiology signature in 7 endomyocardial biopsy samples collected from patients with newly diagnosed cardiomyopathy. The signature performed perfectly in NICM while only one of three ICM samples was classified correctly. This suggests that, compared with NICM patients, those with ICM exhibit greater changes in gene expression as disease progresses. These results parallel those from post-LVAD patients and emphasize the need for stage-specific molecular signatures. To our knowledge, this is the first evidence that microarray hybridization from endomyocardial biopsies is feasible; this success encourages future studies of gene expression signatures using endomyocardial biopsies with RNA amplification.

Prior studies have shown that cardiomyopathy of different etiologies exhibits different patterns of gene expression.[48;51] However, these studies did not develop or prospectively validate a gene expression signature. In fact, one study comparing the gene expression of ICM and NICM found no differentially expressed genes.[47] This study used pooled samples from only two ICM and two NICM patients, and likely did not have adequate resolution to detect changes in gene expression.[102]

Although the differential gene expression between failing and nonfailing hearts has been attributed to age and gender differences,[42] this analysis has not been extended to ICM and NICM. However, we addressed this possibility by stratifying our analysis by clinical characteristics. The sensitivity and specificity were not affected, indicating that the etiology molecular signature's accuracy is not an artifact of differences in baseline characteristics.

Many of the genes in the etiology signature are not known to be expressed in myocardial tissue. This discrepancy has been observed in prior microarray experiments in cardiomyopathy[42;43;47-53;55] and stems from the gap between the number of genes on the microarray platform and our ability to define their functions.[85] However, the inability to justify the biologic validity of every gene in the signature does not invalidate its clinical utility. In prediction analysis, it is the pattern of gene expression, rather than the individual genes themselves, which serves as biomarkers of disease.[57;62;63]

Nevertheless, there is biologic plausibility for a number of genes in the etiology molecular signature. The up-regulation of signal transduction genes in ICM, including several protein phosphatases and a MAP kinase, is supported by evidence that their gene products may protect against ischemic injury.[103-105] The up-regulation of endothelin-converting enzyme in ICM over NICM has also been described.[106] One would expect up-regulation of genes involved in cell growth/maintenance, including ribosomal and cell division cycle proteins, since myocyte proliferation rate is higher in ICM than NICM.[107] However, while these findings support the biologic validity of the etiology signature, the changes bear further investigation with a study focused on differential gene expression.[44]

Gene expression analysis is considered hypothesis-generating until validated by another technique. Unlike the majority of studies in cardiology, where microarray analysis focuses on the discovery of novel genetic pathways, our analysis concentrates on prediction. Thus, validation involves evaluating the predictive accuracy of the signature in independent, blinded samples.[57;59;63] This is an established approach among studies in the cancer literature.[64;66;67] However, the level of transcript abundance should also be confirmed with quantitative PCR to determine if the signature offers utility independent

of the microarray platform. We confirmed the expression level of four genes in the prediction profile using quantitative PCR, and the fold changes agreed in all cases.

Several methodologic aspects of this study warrant mention. There is little information regarding sample size requirements in microarray analysis. One study determined that for accurate class prediction of etiology, a training set of 10-20 samples is required.[102] Thus our sample size was adequate for prediction. We were also able to maximize the amount of information obtained by random partitioning of samples. Furthermore, to our knowledge, this study includes the largest number of samples in a cardiovascular microarray study to date.

Finally, it may be argued that a gene expression signature that identifies patients based on prognosis would be more clinically valuable than one based on etiology, which can be determined by other methods. The current findings are valuable proof-of-concept that other predictions will be possible in the future. Indeed, the transition from gene profiling of etiology[62;99;100] to gene profiling of prognosis,[64-67] represents the path taken in the oncology experience.

This study represents the first use of gene expression signatures in cardiovascular disease and the first evidence that microarray hybridization from endomyocardial biopsies is feasible. Microarray analysis has the potential to optimize the diagnosis and management of patients with myocardial diseases. These results form the basis for future studies using molecular signatures to distinguish cardiomyopathy patients by other relevant clinical parameters. Studies are currently underway to develop gene expression signatures that distinguish ICM and NICM in newly diagnosed patients, as well as to differentiate these patients by prognosis and response to therapy.

Table 3.1 Clinical characteristics of the study subjects used to identify and validate a molecular signature that differentiates between ischemic and nonischemic cardiomyopathy

	End-stage cardiomyopathy		Post-LVAD cardiomyopathy		Newly diagnosed cardiomyopathy	
	Ischemic* (n= 10)	Nonischemic* (n = 15)	Ischemic† (n = 3)	Nonischemic* (n = 13)	Ischemic† (n = 3)	Nonischemic† (n = 4)
Age, y	57.5 (54-60)	46 (37-52) ‡	60 (43-61)	46 (35-51)	63 (57-81)	45 (37-64)
Male	100%	67%§	100%	62%	66%	75%
NYHA classification	4 (3-4)	4 (3-4)	3 (3-4)	3 (3-4)	2.5(2-2.5)	2.8 (2 - 3)‖
Left ventricular ejection fraction, %	18.8 (15.0-25.0)	15.0 (10.0-20.0)	N/A	N/A	20 (12.5-32.5)	20 (15 -27.5)
Left ventricular end-diastolic diameter, cm	6.8 (6.4-7.3)	7.4 (6.8-8.3)	N/A	N/A	4.7 (4.6-5.6)	6.3 (4.9-6.6)‖
Pulmonary artery pressure, mm Hg						
Systolic	49 (35-64)	50 (45-57)	N/A	N/A	47 (46-71)	64 (37-81)
Diastolic	25 (18-33)	30 (24-30)	N/A	N/A	22 (20-36)	30 (19-35)
Pulmonary capillary wedge pressure, mm Hg	27 (14-31)	25 (20-30)	N/A	N/A	17 (15-27)	26 (11-33)
Cardiac index, $L \cdot min^{1} \cdot m^{2}$	2.2 (1.5-2.4)	1.5 (1.3 – 1.9)	N/A	N/A	2.0 (1.4 – 2.3)	1.9 (1.8 – 2.2)
Medications						
Beta antagonists	70%	39%	33%	42%	66%	25%
ACE inhibitors	100%	62%‡	66%	50%	100%	100%
Diuretics	100%	69%	33%	25%	100%	75%
Intravenous inotropic therapy#	10%	62%‡	0%	0%	0%	0%

*Values are median (25^{th} and 75^{th} percentiles) or percentages.

†Values are median (range) or percentages.

‡$p < 0.05$ for end-stage ischemic vs nonischemic cardiomyopathy

§$p = 0.06$ for end-stage ischemic vs nonischemic cardiomyopathy

‖$p < 0.05$ for end-stage vs biopsy subjects and post-LVAD vs biopsy subjects

#Dopamine, dobutamine, or milrinone.

N/A is not available, since routinely, post-LVAD patients do not undergo right heart catherization and ejection fraction lacks clinical relevance in patients receiving mechanical circulatory support.

Table 3.2 Sensitivity and specificity of the etiology molecular signature in strata defined by clinical covariates

	Sensitivity	Specificity
Overall	89%	89%
Age, y		
≥ 50	88%	80%
< 50	100%	90%
Ejection fraction, %		
≥ 15	89%	89%
< 15	100%	83%
Inotropic therapy		
Yes	100%	100%
No	89%	60%

Table 3.3 Genes (n = 90) in the molecular signature that differentiates between ischemic and nonischemic cardiomyopathy

Gene accession no.	Gene symbol	Gene name	Fold change*
Cell growth/maintenance			
AL078621	RPL23AP7	ribosomal protein L23a	2.4
AA086229	ENIGMA	enigma (LIM domain protein)	2.2
NM_005938	MLLT7	myeloid/lymphoid leukemia	2
AA054734	CIZ1	CDKN1A interacting zinc finger protein-1	1.6
AA576621	CDC2L5	cell division cycle 2-like-5	1.5
NM_000076	CDKN1C	cyclin-dependent kinase inhibitor-1C	1.5
NM_003547	HIST1H4G	histone-1	1.5
BC005174	ATF5	activating transcription factor-5	1.4
NM_015487	GEMIN4	gem-associated protein-4	1.4
BC000229	MIS12	homolog of yeast Mis12	-1.5
Cytoskeleton			
U40572	SNTB2	syntrophin, beta-2	1.9
NM_007284	PTK9L	protein tyrosine kinase-9-like	1.8
AI077476	DMN	desmuslin	1.5
NM_014016	SACM1L	SAC1 suppressor of actin mutations-1-like	-1.9
Development			
NM_001420	ELAVL3	Hu antigen C	2.5
AF005081	NA	Homo sapiens skin-specific protein	2
Immune response			
NM_030882	APOL2	apolipoprotein L-2	2.4
NM_030754	SAA2	serum amyloid-A2	2.4
L34163	IGHM	immunoglobulin heavy constant mu	2.3
AA742237	BAT2	HLA-B associated transcript-2	2
Metabolism			
AW134794	SLC39A8	solute carrier family-39, member-8	2.7
AI379894	PPP2CB	protein phosphatase-2 (formerly 2A)	2.2
BC004864	PPP3CC	protein phosphatase-3 (formerly 2B; calcineurin A)	2.2
NM_002779	PSD	pleckstrin and Sec7 domain protein	2.2
NM_006782	ZFPL1	zinc finger protein-like-1	2.2
U94357	GYG2	glycogenin-2	2.1
NM_003456	ZNF205	zinc finger protein-205	2.1

BC005043	MGC31957	hypothetical protein	1.9
NM_014649	SAFB2	scaffold attachment factor-B2	1.8
NM_018135	MRPS18A	mitochondrial ribosomal protein-S18A	1.7
NM_007188	ABCB8	ATP-binding cassette, sub-family-B, member-8	1.6
NM_018411	HR	hairless homolog	1.6
NM_006238	PPARD	peroxisome proliferative-activated receptor, delta	1.6
AA047234	OAZIN	ornithine decarboxylase antizyme inhibitor	1.4
NM_005254	GABPB1	GA binding protein transcription factor	-1.5
NM_015906	TRIM33	tripartite motif-containing-33	-1.6
AL525798	FACL3	fatty-acid-Coenzyme A ligase, long-chain-3	-1.7
NM_004457	FACL3	fatty-acid-Coenzyme A ligase, long-chain-3	-2
Signal transduction			
D10202	PTAFR	platelet-activating factor receptor	2.6
NM_014716	CENTB1	centaurin, beta-1	2.5
BC005365	MAP2K7	mitogen-activated protein kinase-7	2.3
AI860917	PNUTL1	peanut-like 1	2.3
AI688812	RASGRP2	RAS guanyl releasing protein-2	2.3
AF028825	DLG4	discs, large homolog-4	2.2
NM_007327	GRIN1	glutamate receptor, ionotropic,	2.2
NM_006869	CENTA1	centaurin, alpha-1	2.1
AJ133822	AGER	advanced glycosylation end-product-specific receptor	2
NM_007369	RE2	G-protein-coupled receptor	2
AW138374	RHEB	Ras homolog enriched in brain-2	2
X60502	SPN	sialophorin	2
M24900	THRA	thyroid hormone receptor, alpha	2
NM_001397	ECE1	endothelin converting enzyme-1	1.9
L05666	GRIN1	glutamate receptor, ionotropic	1.8
AF287892	SIGLEC8	sialic acid binding Ig-like lectin-8	1.8
NM_014274	TRPV6	transient receptor potential cation channel	1.8
NM_000479	AMH	anti-Mullerian hormone	1.7
NM_014204	BOK	BCL2-related ovarian killer	1.7
U58856	MRC2	mannose receptor, C-type-2	1.6
AI991328	CHK	choline kinase	1.5
NM_000908	NPR3	atrionatriuretic peptide receptor-C	1.4
BG222394	MAPK8IP1	mitogen-activated protein kinase-8 interacting protein-1	1.3
AA460694	KIAA1354	KIAA1354 protein	-1.6

BG111761	GNG12	G protein, gamma-12	-1.8
Transport			
U87555	SCN2B	sodium channel, voltage-gated, typeII	2.1
NM_024681	FLJ12242	hypothetical protein	2
W72053	TGOLN2	trans-golgi network protein-2	-1.6
AJ131244	SEC24A	SEC24 related gene family, member A	-2
Other			
AK025352	MAST205	serine/threonine protein kinase	2.3
AI818951	MGC40499	hypothetical protein	2.3
AK025188	FLJ20699	hypothetical protein	2.2
AI831055	SFTPC	surfactant protein C	2.2
BC004264	EPHB4	ephrin receptor	2.1
NM_031304	MGC4293	hypothetical protein	2.1
D38024	DUX4	double homeobox-4	1.9
NM_003061	SLIT1	slit homolog-1	1.9
NM_024821	FLJ22349	hypothetical protein	1.8
NM_019858	GRCA	likely ortholog of mouse gene rich cluster	1.8
AF023203	NA	Homo sapiens homeobox protein	1.8
NM_030935	THG-1	TSC-22-like	1.8
NM_025268	MGC4659	hypothetical protein	1.6
BC000979	DDX49	DEAD (Asp-Glu-Ala-Asp) box polypeptide-49	1.5
AK021505	NA	Homo sapiens cDNA clone	1.5
NM_018049	GNRPX	likely ortholog of mouse guanine nucleotide releasing protein	1.4
AA018777	NA	ESTs	1.2
AF052151	MTVR1	Mouse Mammary Turmor Virus Receptor homolog-1	-1.3
AL525412	MYCBP	Mycbp-associated protein	-1.4
NM_012311	KIN	antigenic determinant of recA protein	-1.5
NM_018553	HSA277841	ELG protein	-1.6
AA191576	NPM1	nucleophosmin	-1.6
NM_016628	WAC	WW domain-containing adapter with a coiled-coil region	-1.8

*Fold change is mean expression for ischemic relative to nonischemic samples.

Figure 3.1 Study design to identify and validate a molecular signature that differentiates between ischemic and nonischemic cardiomyopathy. The samples were first divided into a training set of end-stage samples from Johns Hopkins Hospital (JHH) used to develop the etiology signature. It was then validated by determining its sensitivity and specificity in three test sets: end-stage samples from the University of Minnesota (UM), post-LVAD samples from both JHH and UM, and endomyocardial biopsy samples from newly diagnosed patients at JHH. Then the end-stage samples were randomly partitioned into training and test sets to identify the representative etiology signature and the overall sensitivity and specificity.

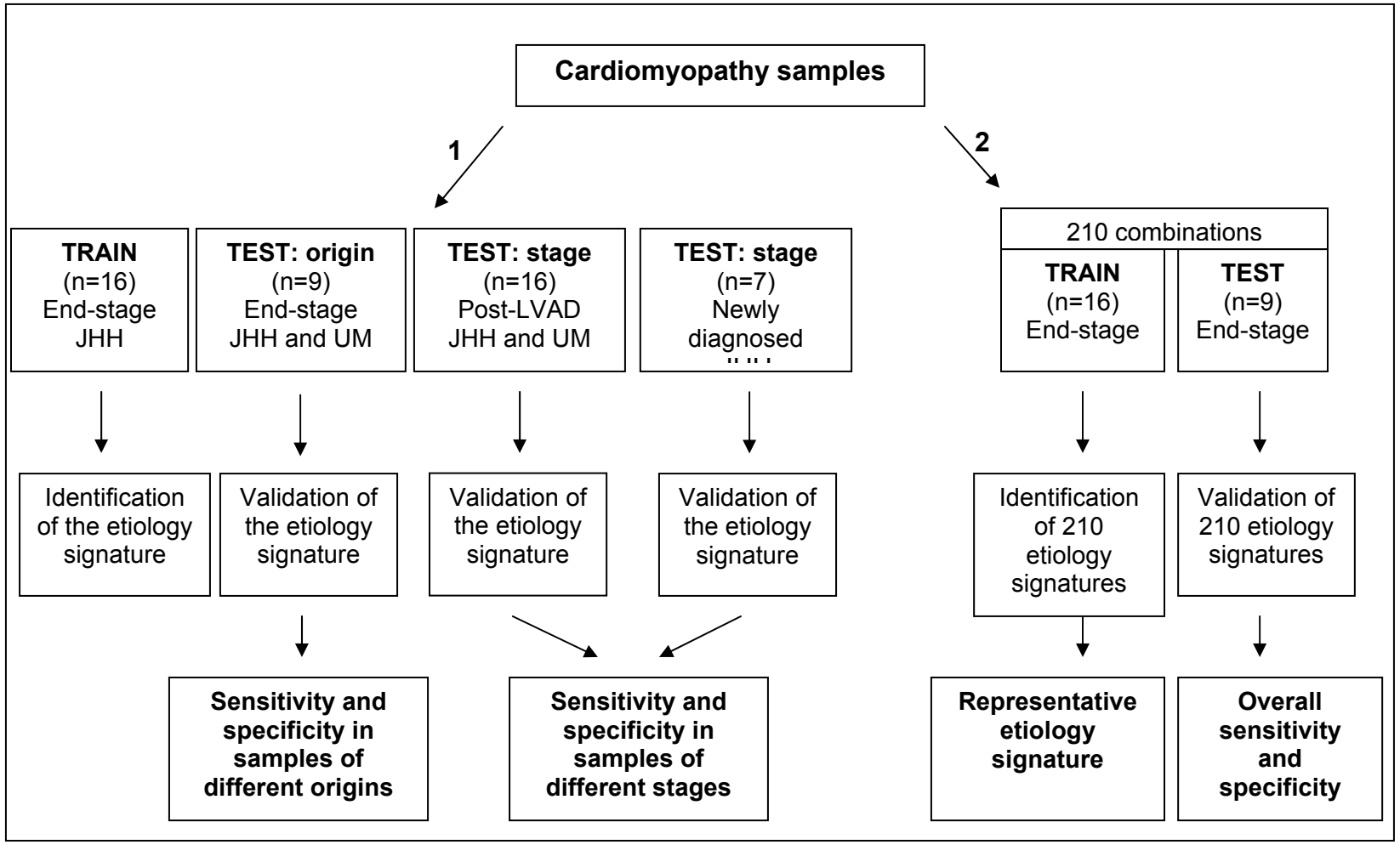

Cardiomyopathy samples
1
2
TRAIN
(n=16)
End-stage
JHH
TEST: origin
(n=9)
End-stage
JHH and UM
TEST: stage
(n=16)
Post-LVAD
JHH and UM
TEST: stage
(n=7)
Newly
diagnosed
210 combinations
TRAIN
(n=16)
End-stage
TEST
(n=9)
End-stage
Identification of the etiology signature
Validation of the etiology signature
Validation of the etiology signature
Validation of the etiology signature
Identification of 210 etiology signatures
Validation of 210 etiology signatures
Sensitivity and specificity in samples of different origins
Sensitivity and specificity in samples of different stages
Representative etiology signature
Overall sensitivity and specificity

Figure 3.2 Number of genes from the etiology molecular signature that are up- and down-regulated in ischemic cardiomyopathy relative to nonischemic cardiomyopathy classified by functional group. CGM is cell growth/maintenance, CYT is cytoskeleton, DEV is development, IMM is immune response, MET is metabolism, OTH is other, SIG is signal transduction, and TRA is transport.

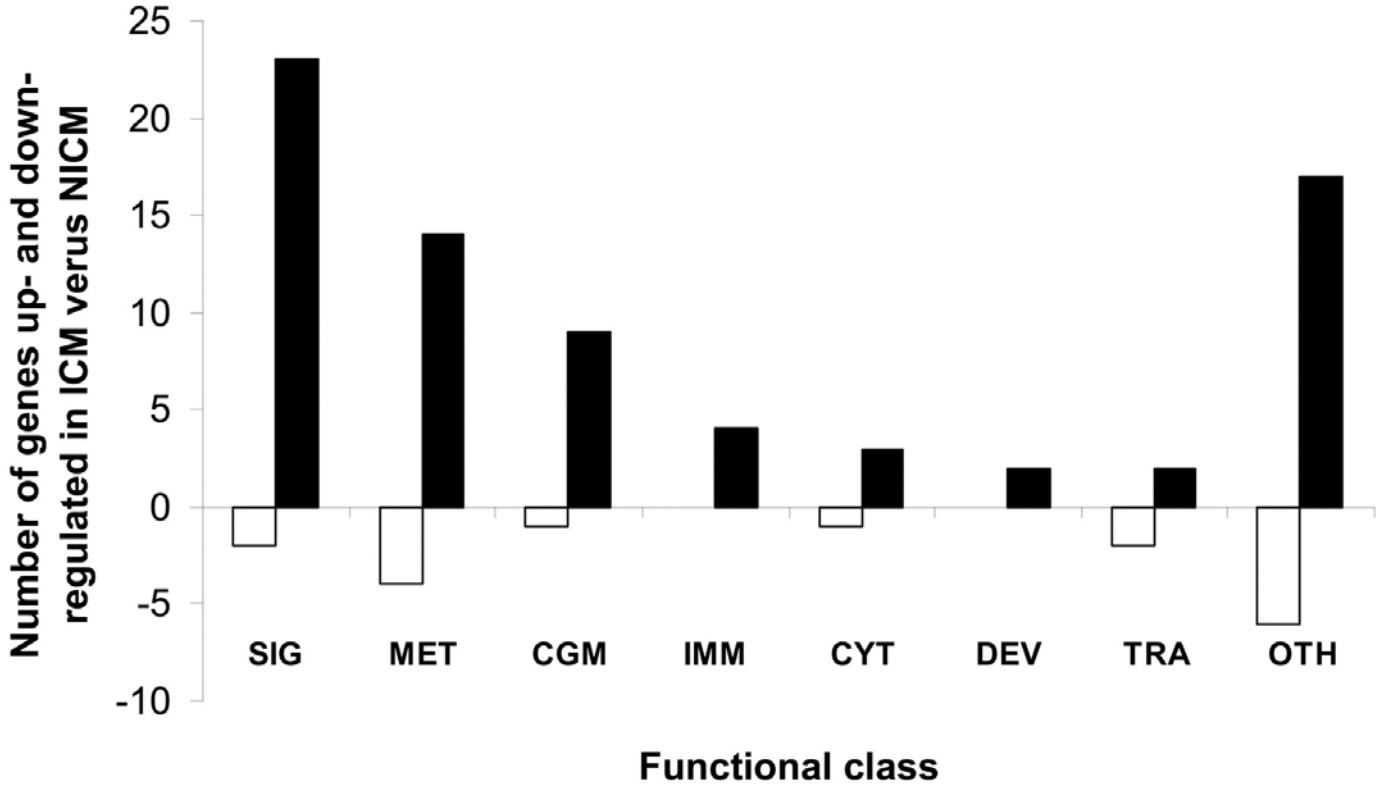


Figure 3.3 Hierarchical clustering of genes that comprise the etiology molecular signature. Each row represents a gene and each column represents a sample. The color in each cell reflects the level of expression of the corresponding gene in the corresponding sample, relative to its mean level of expression in the entire set of samples. Expression levels greater than the mean are shaded in blue, and those below the mean are shaded in red. The samples form two distinct clusters based on etiology. Arrows denote samples that do not appear in their etiology cluster. ICM denotes end-stage or post-LVAD ischemic cardiomyopathy and NICM denotes end-stage or post-LVAD nonischemic cardiomyopathy. ICMB denotes newly diagnosed ischemic cardiomyopathy and NICMB denotes newly diagnosed ischemic cardiomyopathy

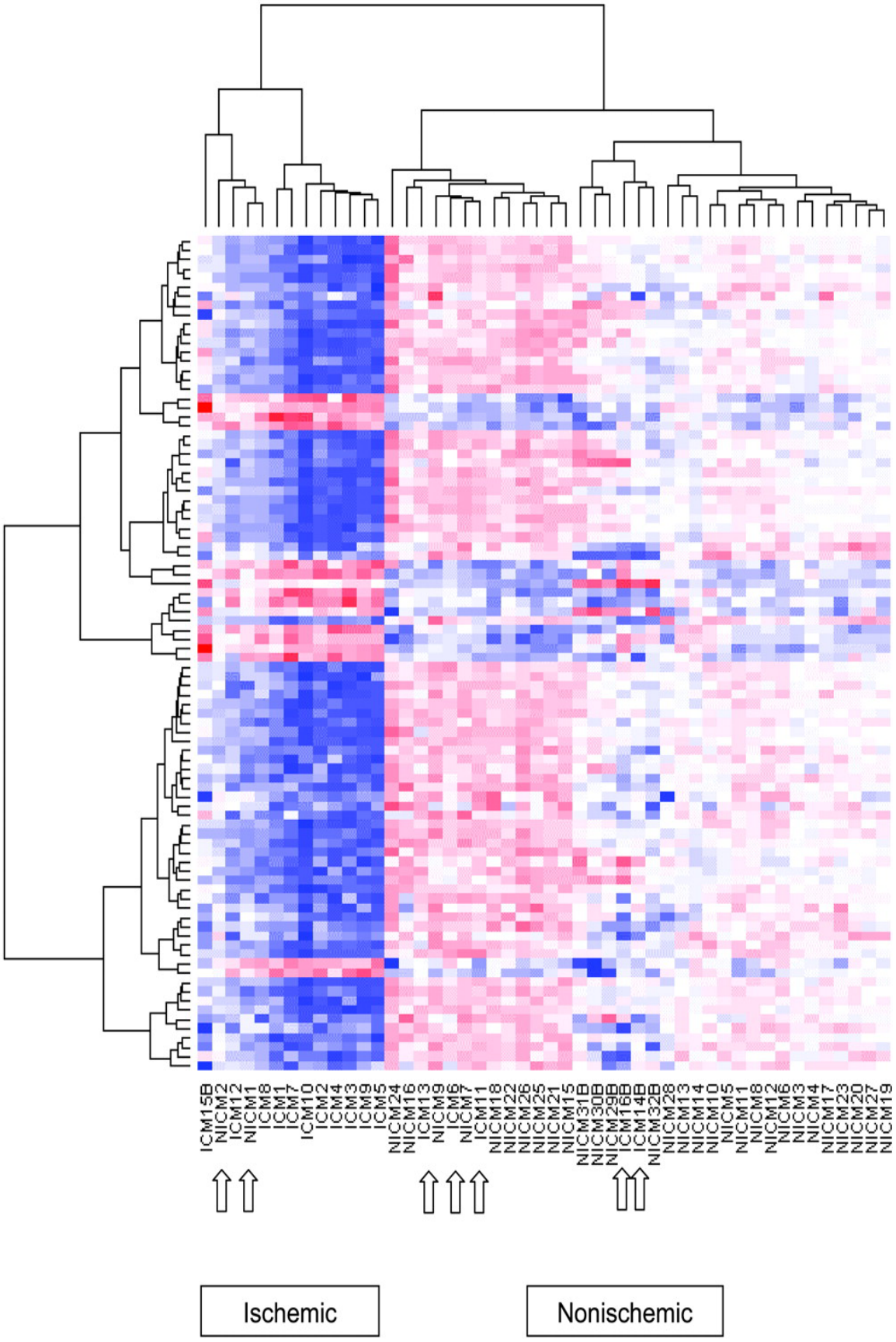
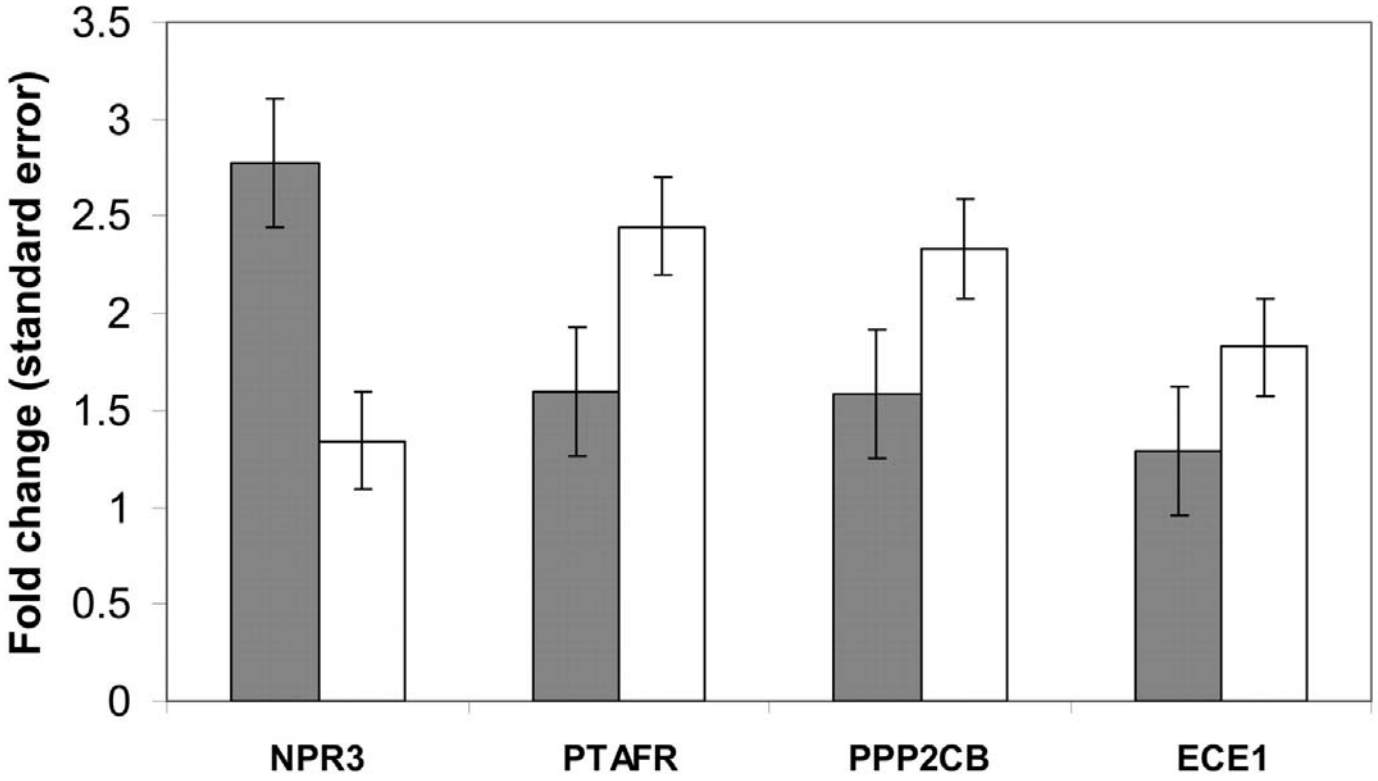

ICM15B
NICM2
ICM12
NICM1
ICM8
ICM1
ICM7
ICM10
ICM2
ICM4
ICM3
ICM9
ICM5
NICM24
NICM16
ICM13
NICM9
ICM6
NICM7
ICM11
ICM18
NICM22
NICM26
NICM25
NICM21
NICM15
NICM31B
NICM30B
NICM29B
ICM16B
ICM14B
NICM32B
NICM28
NICM13
NICM14
NICM10
NICM5
NICM11
NICM8
NICM12
NICM6
NICM3
NICM4
NICM17
NICM23
NICM20
NICM27
NICM19
Ischemic
Nonischemic

Figure 3. Independent assessment of gene expression levels by quantitative PCR for select genes in the etiology molecular signature. Fold change in expression is in ischemic relative to nonischemic hearts according to qPCR (gray bars) and microarrays (white bars) with standard errors. Gene symbols are those from Table 3.



Chapter 4

Conclusions and Future Perspectives

*Adapted from:

Kittleson MM, Hare JM. Molecular Signature Analysis: Using the Myocardial Transcriptome as a Biomarker in Cardiovascular Disease. *Trends Cardiovasc Med* 2005; 15:130-8. With permission from Elsevier.

Kittleson MM, Hare JM. Molecular signature analysis: The potential of gene expression analysis in cardiomyopathy. *Future Cardiology* 2005; 1: 793-808. With permission from Future Medicine, Ltd.

Lessons learned: gene discovery versus molecular signature analysis

The pitfalls of gene discovery

The two studies contained in this dissertation highlight the complementary yet distinct goals and statistical methods involved in gene discovery and molecular signature analysis. Both demonstrate that unique gene expression exists in the two major forms of cardiomyopathy. On one hand, the unique patterns of gene expression can provide insight into potential cause-specific therapies for heart failure. On the other hand, the unique patterns of gene expression can function as a diagnostic biomarker. These two analyses are not redundant, since they used different sets of samples, different statistical methods, and most importantly, had different purposes. Given this, it is not surprising that only four of the identified genes were shared between the two studies. However, this lack of overlap illustrates a troubling aspect of analyses focused on gene discovery: how reliable and biologically valid are the results? These are the two major pitfalls of gene discovery.

The problems with reliability are highlighted in Table 2.5. This table illustrates that there is remarkable congruence in the direction and magnitude of differential gene expression between published studies of gene discovery in failing and nonfailing hearts. However, despite this agreement, these genes represent the minority of genes identified in the studies; the majority of differentially expressed genes identified do not overlap, and how to resolve this variability between studies is unclear.

Analyses of gene discovery also highlight troubling questions regarding biological validity. Two studies attempted to validate the differentially expressed genes by confirming not only levels of transcript abundance, but also their corresponding

protein products. However, in both studies, there was little agreement between transcript abundance measured on the microarray and the corresponding proteins.[53;54] On one hand, this lack of agreement is not surprising, since a number of factors affect the measured protein abundance, including differences in mRNA localization, processing, stability, translation efficiency, as well as posttranslational protein modification and interactions. However, while the lack of agreement is not surprising, it is concerning. Since cellular processes are mainly mediated by proteins, mRNA changes unaccompanied by corresponding alterations in protein may not be meaningful. Furthermore, even if protein abundance confirms the gene expression levels noted by microarray analysis, a biological role for these changes has still not been established.

While there is no clear consensus on how to resolve these problems of reliability and biological validity in microarray studies focused on gene discovery, one point is clear: the role and value of validation differs between gene discovery and molecular signature analysis.

The role of validation

Studies of gene discovery have significant merit, in offering valuable hypothesis-generating insight into possible mechanistic pathways. Nevertheless, results must be interpreted with caution, and confirmation of transcript abundance via a complementary method should be only the first step in validating the significance of the differentially expressed genes. The potential novel genetic pathways or targets of cause-specific therapies must be further elucidated with studies focused on establishing causality, such as *in vitro* or animal models of deletion or over-expression of the target gene product.

In contrast, microarray analyses focused on molecular signature analyses are less vulnerable to problems with reliability and biological validity because their utility is confirmed by testing the molecular signature's predictive accuracy in independent samples. Molecular signature analysis is based upon a *pattern* of gene expression rather than the *identity* of specific genes.[59] The prediction algorithm is able to compare an unknown sample and determine how closely it resembles one pattern versus the other; the absolute expression of an individual gene carries relatively small weight compared to the overall signature. Thus, validation in molecular signature analysis cannot solely involve the confirmation of gene expression levels via a complementary technique. Nevertheless, confirmation of transcript abundance can prove useful to address a different issue: whether the molecular signature offers utility independent of the microarray platform used to create it. This is important if disease-specific platforms are developed, as in the oncology experience.[108;109]

Lessons learned: technical issues in gene expression analysis

Information management and reproducibility in microarray experiments

Given the rapid growth of microarray research and the vast amount of information that can be gleaned from a single experiment, there are many challenges in designing studies and interpreting the results. To this end, the Microarray Gene Expression Data society (MGED) has developed the Minimum Information About a Microarray Experiment (MIAME) standards that are needed to enable the interpretation of the results of the experiment unambiguously and potentially to reproduce the experiment.[110] MIAME includes details of the experimental design, sample preparation, hybridization procedures, normalization algorithms, and array design. The ultimate goal is to establish

a standard for recording and reporting microarray-based gene expression data, which will in turn facilitate the establishment of databases and public repositories and enable the development of data analysis tools.

Such public repositories exist, including the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO); the European Bioinformatics Institute (EMBL-EBI) ArrayExpress repository; and the Center for Information Biology Gene Expression (CIBEX) database. There is also a cardiology-specific repository, the Cardiogenomics website (www.cardiogenomics.com), a National Heart Lung and Blood Institute-sponsored Program for Genomic Applications. Many journals currently require that submissions comply with the MIAME standard, but not all require that a complete data set be submitted to one of three databases prior to manuscripts submission. Furthermore, the public repositories do not require that the data be submitted in raw as well as normalized fashion, which would be essential for future studies. Mandatory submission of raw microarray data files to public repositories, by promoting collaboration among scientists, would be essential for maximizing the utility of microarray research.

Sample size in microarray experiments

Clearly, molecular signature analysis is in its earliest stages in cardiomyopathy, and there are many unresolved issues. For example, there is limited knowledge on the sample size required in microarray experiments. The largest microarray study in cardiomyopathy to date has involved 199 samples,[54] and in the oncology literature, each study has employed fewer than 300 patients. However, this could be considered a strength of these analyses: a succession of smaller studies, performed quickly and with the use of improving technology, may surpass larger studies locked into out-of-date

approaches.[63] Furthermore, by randomly partitioning samples as we did in identifying and validating a gene expression signature, it is possible to maximize the utility of a limited number of samples.

The source of tissue for analysis

To date, microarray analyses in cardiomyopathy have mainly utilized discarded myocardial tissue obtained at the time of cardiac transplantation or LVAD placement, and there are limitations to this approach. First, the tissue is obtained from patients late in the disease course and thus the conclusions may not be applicable to patients at an earlier stage of disease; as we demonstrated, a molecular signature based on etiology in end-stage cardiomyopathy is specific to disease stage.[75] Second, explanted tissue is obtained from different areas of the left ventricle, and there is evidence from microarray analysis in mice that regional differences in gene expression exist in the left ventricle.[111] Thus, in the future, microarray analyses in cardiomyopathy will ideally focus on endomyocardial biopsy tissue obtained from patients at earlier stages of disease, and we have demonstrated that microarray hybridization from endomyocardial biopsies is feasible.[75]

Although one commonly invoked limitation of gene expression research in cardiovascular disease is the lack of ready access to human heart tissue samples, endomyocardial biopsies are frequently performed to evaluate newly diagnosed cardiomyopathy.[29;112] Endomyocardial biopsy is a safe and well-tolerated procedure, with an overall mortality rate of 0.2%,[113] a rate equivalent to that of other routinely performed catheterization procedures.

While endomyocardial biopsy could be more widely performed if a valuable prognostic or diagnostic test were developed, venipuncture is clearly more accessible. Therefore, it is essential to also test the utility of molecular signature analysis in peripheral blood leukocyte samples. In the cancer literature, molecular signatures derived from peripheral blood leukocytes offer comparable predictive accuracy to those from solid tumor samples in classifying subjects by cancer type and type of therapy.[114;115] This may also be feasible in cardiovascular disease, as peripheral blood molecular signatures correlated with biopsy-proven allograft rejection in cardiac transplant recipients[116] and cardiac genes in circulating blood are differentially expressed in patients with coronary artery disease relative to controls without heart disease.[117]

Future perspectives

Prognosis

Knowledge of prognosis is essential for patient education and allocation of therapies, especially those that may be costly and invasive. In the future, it may be possible for a patient with a newly diagnosed cardiomyopathy to better understand, through an assessment of his or her "prognostic molecular signature," whether his or her ejection fraction and functional status will improve, or whether he or she will go on to develop circulatory collapse. This knowledge may then be used to pursue more aggressive management in patients who will do poorly, including earlier evaluation for heart transplantation or ventricular assist device placement.

Individualization of patient management

There is also evidence that gene expression may differ based on response to therapy in heart failure. In a study using candidate gene expression analysis, patients

with idiopathic dilated cardiomyopathy who improve their ejection fraction in response to treatment with beta-blockers had associated changes in the expression of myocardial contractility-regulating and hypertrophy-associated genes.[118] This lends support to the development of molecular signatures that predict which patients will derive benefit from heart failure therapies.

Another key area where this technology will be helpful is in deciding to pursue therapies that may be risky, costly, and invasive, and the implantable cardiac defibrillator (ICD) is a prime example. Although there is clear mortality benefit to ICD implantation in all patients with an ejection fraction less than 35%,[19] the current consensus is that we have not yet identified subsets of high-risk individuals who will benefit most from this therapy.[119;120] In the future, it may be possible to identify a "sudden cardiac death molecular signature," that identifies cardiomyopathy patients who are at high risk for this manner of death.

In addition, it would be very useful to predict which patient may have an adverse response to therapy. For example, 3 to 5% of patients receiving anthracycline chemotherapy will develop a severe, symptomatic cardiomyopathy, which is usually irreversible and may subsequently require cardiac transplantation.[121] Although risk factors exist, it is currently not possible to predict which patients will sustain serious cardiac damage and develop heart failure. However, if a gene expression profile could predict which patients undergoing chemotherapy were at increased risk for anthracycline-induced cardiomyopathy, chemotherapeutic regimens could be tailored to prevent this devastating complication of cancer therapy.

With this evidence of heterogeneity in response to therapy and an association between response to therapy and gene expression, we envision developments over the next decade in which gene expression-based molecular signatures are used to tailor therapies for heart failure patients. Molecular signatures could also be incorporated into clinical trials of new drugs in order to identify those patients who demonstrate the greatest benefit or harm from a given therapy. Using endomyocardial biopsy tissue or even peripheral blood leukocytes, it may be possible to identify a patient's molecular signature that will determine their prognosis and response to therapy.

Conclusions

Given the vast array of pharmacologic, device, and surgical therapies for heart failure, there are endless examples in which the individualization of management in cardiomyopathy patients would be helpful. With the promise of this technology, it is essential understand the opportunities and pitfalls of this burgeoning field, and to design future studies to address these important issues.

The most powerful and promising application of gene expression profiling with microarrays is molecular signature analysis, where a pattern of genes identified by the microarray analysis is used as a biomarker to identify or predict a clinically relevant parameter. Molecular signature analysis could augment current clinical and imaging modalities used to determine the prognosis and response to therapy of dilated cardiomyopathy patients. There is evidence that this is a feasible strategy in cardiomyopathy, with our study identifying and validating a molecular signature that differentiates cardiomyopathy patients by etiology.

Although there are unresolved issues in this field, including sample size requirements and the source of tissue for analysis, there is still much promise. The ultimate goal of molecular signature analysis will be to contribute towards an individualization of the management of heart failure patients, whereby a patient with a newly diagnosed cardiomyopathy could, through gene expression analysis, be offered an accurate assessment of prognosis, and how individualized medical therapy could affect his or her outcome.

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Curriculum vita

Michelle Maya Kittleson was born in Bridgeport, Connecticut on May 23, 1974. She graduated Harvard College as an Advanced Standing Scholar in 1995, receiving a degree in biochemical sciences in three years instead of four. Her honors thesis, under Dr. Thomas Benjamin in the Department of Pathology at Harvard Medical School, focused on the size-exclusion chromatographic properties of polyomavirus middle T antigen. In college, she was honored with the Detur Prize, given to students in the top 0.5% of the freshman class.

Dr. Kittleson received her M.D. from the Yale University School of Medicine in 1999. She completed her medical school thesis under the guidance of Dr. Carlos Grilo at the Yale Psychiatric Institute where she focused on the reliability and validity of a diagnostic questionnaire for psychiatric inpatients, and received the Parker Prize, granted to the medical student most qualified as a physician.

Dr. Kittleson completed an internship and residency in internal medicine at Brigham and Women's Hospital in 2002. She was honored with the Arnold Dunne Award, given to those interns who show extraordinary care and caring for their patients. Dr. Kittleson also spent six months of her senior year working with Dr. Lynne Stevenson, examining the clinical characteristics and mortality of heart failure patients intolerant of ACE inhibitors due to circulatory or renal limitations.

Currently, Dr. Kittleson is in her fourth year of clinical cardiology training at Johns Hopkins. She enrolled in the Graduate Training Program in Clinical Investigation (GTPCI) at the Johns Hopkins Bloomberg School of Public Health in 2003. She has been

honored with the Clinical Researcher Annual International Award, bestowed upon the student with the highest score on the GTPCI comprehensive examination, as well as the Samuel A. Levine Young Clinical Investigator Award from the American Heart Association, the Pearl M. Stetler Research Fund for Women Physicians Fellowship Award, and the Frederik B. Bang Award for Student Research. During her fellowship, she has co-authored five original research articles as first author, six abstracts, three review articles, four editorials, and a book chapter. From a clinical standpoint, Dr. Kittleson has completed specialty training in heart failure and cardiac transplantation. Her broad research experience has solidified her desire to have an academic career in cardiology, and her current research focuses on gene expression analysis and other novel biomarkers in the management of cardiomyopathy and cardiac transplantation.

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